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Water and asexual reproduction in the ingoldian fungi

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Resumen

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Con el fin de primero definir el término hongos ingoldianos se revisan brevemente los diversos grupos biológicos de hongos filamentosos que se hallan en ambientes acuáticos. Los términos que definen a estos grupos están basados en criterios fisiológicos, ecológicos o taxonómicos. Los dos primeros (que distinguen entre acuático y terrestre) no están debidamente definidos y crean confusión. Por otro lado, los criterios taxonómicos basados en morfología están bien definidos y son por tanto útiles por lo menos a nivel de morfos. Se detectan en la naturaleza seis morfos, cinco de los cuales son fácilmente reconocidos. A nivel de especie, sin embargo, estos criterios no pueden ser tan fácilmente aplicados porque los ciclos vitales no son siempre bien conocidos. Se comenta el término «hongos ingoldianos» en este contexto.

Se cree que las dificultades observadas para la producción *in vitro* de conidios en los hongos ingoldianos se deben a un conocimiento deficiente de las relaciones del hongo con su medio acuático. Hemos por tanto revisado la literatura en busca de factores ambientales, tanto en condiciones de campo como de laboratorio, que afecten a la reproducción asexual de estos hongos, tanto a nivel de micelios individuales como al de comunidades.

Entre las propiedades reproductivas del micelio afectadas por el agua tenemos en cuenta: la concurrencia del crecimiento vegetativo con la esporulación, madurez fisiológica, esterilidad de las colonias, crecimiento restringido, esporulación «sumergida» y comportamiento infraespecífico, así como caracteres que afectan a la cuantificación con fines experimentales: adherencia y flotabilidad de conidios, conidiación microcíclica, esporulación secundaria y fragmentación conidial. A nivel de comunidades, se comentan interacciones con otras especies de hongos ingoldianos, otros hongos u otros microorganismos, principalmente bacterias.

Los factores ambientales revisados se dividen en ecológicos (o sea hábitats) y fisiológicos. Los hábitats pueden estar o no estar sumergidos. Los no sumergidos corresponden a partes aéreas o subterráneas de plantas vivas terrestres, a la hojarasca vegetal y al suelo. Los hábitats sumergidos pueden ser aguas con diferentes grados de salinidad o bien aguas dulces. Entre éstas se distingue entre aguas leníticas y lóticas, pudiendo ser las últimas permanentes, incluyendo ríos de gran caudal, o temporales. Se comenta brevemente el efecto de la contaminación orgánica sobre la esporulación en aguas lóticas.

Se describen dos factores fisiológicos primordiales: la naturaleza de los sustratos (incluyendo el efecto de las lesiones miceliares en la esporulación) y la del medio líquido circundante, y dentro de estos se comenta el efecto de nutrientes disueltos y su renovación, actividad acuática, intercambio de gases, mezclado y renovación del agua y presión hidrostática. Dos importantes fenómenos observados en los ríos han sido poco estudiados: represión de la esporulación sumergida y proliferación de conidios en períodos de crecida.

En tercer lugar se revisan las técnicas usadas para la producción de conidios. Se basan en la creación de dos ambientes: aire húmedo y presencia de medios líquidos. Estos pueden ser agua pura o soluciones nutritivas débiles. El agua se usa sin cambiar (ya sea estática o turbulenta), o bien se cambia periódica o continuamente, en ambos casos con o sin aireación forzada. Las soluciones nutritivas se usan sin cambiar o bien renovadas periódica o continuamente, y en este caso se expone el sustrato al aire o bien se sumerge, con intercambio de gases forzado o pasivo (por difusión). Algunas técnicas basadas en el uso de soluciones nutritivas se están poniendo a prueba en nuestro laboratorio, y se presentan resultados preliminares.

Se concluye el trabajo con una propuesta de lineas de investigación a partir de los diversos temas cubiertos arriba.

Abstract

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A number of biological groups are being proposed in the literature for filamentous fungi found in continental aquatic habitats. Terms defining them are based on physiological, ecological and taxonomic criteria. The first two (which distinguish between aquatic and terrestrial) are not yet properly defined and create confusion. Taxonomic criteria based on morphology, on the other hand, are well defined and thus useful at least as applied to morphs. Six morphs are thus easily recognized. At the species level, however, such criteria are not as easily applied, as life cycles are not always known. The term Ingoldian fungi is discussed in this context.

Difficulties encountered in *in vitro* conidial production in the Ingoldian fungi are believed to be largely caused by our poor understanding of water relations. We have thus surveyed the literature for relevant environmental factors, both in the field and under controlled conditions, affecting asexual reproduction of these fungi as individual mycelia or at the community level.

Among the reproductive properties of individual mycelia affected by water relations we consider: concurrence of vegetative growth and sporulation, reproductive maturity, staling, restricted growth, «submerged» sporulation and infraspecific behaviour, as well as some characters affecting quantification for experimental purposes: conidial stickiness and buoyancy, microcycle conidiation, secondary sporulation and conidial fragmentation. At the community level, we refer to interactions with other Ingoldian fungi, other fungi or other microorganisms, mainly bacteria.

Environmental factors have been broken down into ecological (i.e. habitats) and physiological. Habitats are treated as either non-submerged or submerged. Non-submerged ones can be aerial and underground parts of live terrestrial plants, plant litter and soil. Submerged habitats include waters with various degrees of salinity. Within fresh waters we discuss lentic and lotic ones, and these refer to permanent streams (including little-studied large rivers) and temporary streams. The effect of organic pollution on sporulation in lotic waters is briefly discussed.

Physiological factors discussed are: the nature of substrates (including the effects of wounding) and that of the surrounding liquid medium, i.e. dissolved nutrients and their renewal, water activity, gaseous exchanges, water mixing (or turbulence) and renewal and hydrostatic pressure. Two little understood features of field sporulation are highlighted, i.e.: suppressed sporulation and increased conidial loads in spate.

Techniques used for conidial production are surveyed. Two basic environments are created: moist air and contact with liquids. The latter may be distilled water or weak nutrient solutions. Water may be used unchanged, and then kept standing or mixed, or it is changed either periodically or continuously, in both cases with or without forcible aeration. Nutrient solutions are left unchanged or they are renewed either periodically or continuously, and then either exposed to air or submerged with forcible or passive gaseous exchange. Preliminary results are presented on some of the techniques using continuous supply of nutrient solutions, and which are being tested in our laboratory.

Finally, various lines of research are proposed on the basis of the above discussion.

INTRODUCTION

Groups of filamentous fungi in terrestrial waters

In order to obtain a realistic picture of the biological groups of filamentous fungi present in terrestrial aquatic habitats and their ecotones, we must first of all identify and name all reproductive structures (morphs), either directly on the substrates or in pure culture. Secondly, there is a need to find, also through pure culture, the possible genetic connections between these morphs, i.e. those between anamorphs (if one or more of these are present) and especially between anamorphs and teleomorphs.

Basically five types of morphs of filamentous fungi can be readily recognized in terrestrial aquatic habitats:

1. Ascomycetous teleomorphs: around 340 belong to typically «freshwater or aquatic Ascomycetes» (see Shearer, 1993 and Goh & Hyde, 1996). Ascospores are found in running waters, but only a few (e.g. leptosphaeriaceous and some lichenized forms) are morphologically distinguishable as such, and even fewer at the species level.
2. Basidiomycetous teleomorphs: although apparently not as abundant as the ascomycetes, small corticiaceous and even delicate agaricaceous forms always appear when substrates from streams are moist incubated, and larger forms (obviously needing a greater mycelial biomass) are occasionally seen fruiting on partly submerged branches and trunks, or for example in wood piles. An aquatic (or amphibious) basidiomycetous group parallel to that of the aquatic ascomycetes has however not yet been proposed. Although waterborne basidiospores are generally less differentiated than ascospores and cannot be recognized in water or foam samples, they are probably present here too. None are known to be recognizable at the species level.

3. Hyphomycetous anamorphs, which are mostly moniliaceous. A strikingly high number are stauroconidial, and far fewer are scolecoconidial. Around 270 morphs of these two types can now be named. Stauroconidia found in water are often recognizable at the species level. Many species are still only known as conidia in water. An unknown but certainly large number of hyphomycetous anamorphs associated with terrestrial waters bear little-differentiated, unbranched conidia (and belong for example to the «geofungi» discussed below), but they have not yet been treated as a distinctly aquatic group. Their conidia are not as a rule species-diagnostic.

4. Basidiomycetous anamorphs: branched or scolecoid conidia outwardly similar to those of hyphomycetous ones but with dolipore septa and dikaryotic cells, are regularly found in streams. (Monokaryotic conidia could also be present in nature but there is sofar no evidence for monokaryotic mycelia). Clamp connections are not produced by conidia of all species. We have no records of basidiomycetous anamorphs with non-differentiated conidia, but they are most probably present too.

5. Coelomycetous anamorphs are extremely abundant in streams but practically ignored. Many are ameroconidial, e.g. *Phoma*-like, and thus difficult to identify to species morphologically. Some species with conidia bearing filiform appendages have been reported, but there are few with branches as broad as the axis (e.g. *Tetranacrium*, known only from lentic habitats).

6. Clamped and *Rhizoctonia*-like mycelia can be seen on leaves and wood, and could be considered as anamorphs, and they are not identifiable to species morphologically, unless cultured.

In situ assignation of the above morphs to true biological species is convenient not only for taxonomic purposes (e.g. biodiversity studies), but also for ecological application. This is, however, not always so simple:

a) Hyphomycetous ascomycete morphs are normally considered to have a dispersive (or propagative) function; but some, especially those with tiny ameroconidia, are presumed to be spermatial states of either Ascomycetes (e.g. of *Hymenoscyphus* in the Leotiales) or of so far pleo-anamorphic fungi with stauro- or scolecoconidia. *Anguillospora rosea* (Descals *et al*. 1998), has scolecoid microconidia which may be spermatial, as they have been seen fusing with undifferentiated hyphae in what is presumably a stage in sexual reproduction ispermatial and dispersive functions could be combined in the same conidium (as happens in the terrestrial *Chrysonilia*).There is at least one precedent of a probably dispersive synanamorph (a *Tricellula* indistinguishable from *T. aquatica*) associated with an aerially produced morph with larger scolecoconidia, *Spermospora lolii* (MacGarvie & O'Rourke 1969).

b) Some coelomycetous morphs with ameroconidia produced in pycnidia are most probably spermogonia of dothideaceous ascomycetes, some of which have been described (e.g. *Massarina* spp. with *Clavariopsis, Tumularia* and *Anguillospora* hyphomycetous anamorphs).

c) Ascomycetous morphs may be genetically connected to hyphomycetous or coelomycetous anamorphs, some of which have been already described.

d) Basidiomycetous morphs could be the teleomorphs of basidiomycetous or

even of apparently hyphomycetous dispersive anamorphs, of which some again have been described.

e) Plating techniques applied to submerged leaves yield a significant number of sterile mycelia, of which nothing is known. Woody substrates should probably be worth studying in this context.

A number of names have been proposed for filamentous fungi found in continental aquatic habitats, e.g. litter and soil fungi (presumed to be only transients), facultative aquatic hyphomycetes, aero-aquatic hyphomycetes or fungi, aquatic hyphomycetes, amphibious hyphomycetes, terrestrial aquatic fungi, terrestrial Ingoldian hyphomycetes, submerged aquatic hyphomycetes, aquatic ascomycetes, Ingoldian fungi, etc.

These terms (except that of Ingoldian fungi) can have two components: one component attempts to empharize fungal relationships with water (e.g. aquatic, aero-aquatic). (Other possible environmental parameters are curiously ignored). The other component is taxonomic (e.g. hyphomycetes, ascomycetes or just fungi). Criteria used for water relationships are however not clear. They are used in a physiological sense, but then may not distinguish between vegetative growth, sexual or asexual reproduction, or else they do not consider physiochemical aspects of the aquatic medium in which fungi live or reproduce. If used in an ecological sense, they may confuse for example residence with dispersal, or else the presence of ecotones is ignored. The taxonomic classification of many groups of microfungi is currently unstable, as they are being constantly affected by the slow but unrelenting discovery of new anamorph-teleomorph connections, and will soon probably also be affected by clarifications of relationships at the molecular level.

The terminological confusion which is being created by the lack of clear criteria for defining the above biological groups can interfere with our understanding of reproductive processes and with the interpretation of environmental conditions affecting those. It may be advisable at the moment to refrain from proposing new biological groups, or even from using some of the published ones. We should instead try to find some solid bases for a better understanding of fungal reproduction in terrestrial waters.

Physiological or ecological criteria must be analyzed by experts in these fields. For the purposes of this paper we need some working definitions: aquatic (asexual) sporulation will apply to that taking place in contact with free water or nutrient solutions (either submerged or at the air-water interface, or just «interface» for brevity's sake); and aerial sporulation will refer to that taking place on unsubmerged mycelia, whatever the relative humidity may be. The latter may however eventually need further refinement, as here conidia may be formed in condensation droplets and thus approach interface or submerged conditions. We should also remember that water activity and other physiochemical factors affecting both the substratum and the liquid medium probably have a strong influence on whether a fungus sporulates above, at or below the water surface.

Some of the more relevant biological groups mentioned above need to be discussed in order to circumscribe better the term Ingoldian fungi used in our title:

a) Aquatic hyphomycetes and amphibious hyphomycetes

In July of 1940, while searching for chytrids, Ingold discovered a variety of highly differentiated spores «suspended in the water of a small stream» in England (Ingold 1942). Unfortunately, Ingold did not discuss sampling techniques and we can only guess that foam was the source for his spores. It is apparently not until ten years later that Ingold & Ellis (1952) specifically mentioned scum when they wrote: «In December 1950 one of us (E.A.E.) encountered a collection of fungus spores in the scum on the surface of a fresh-water tidal ditch...». Foam in streams was first cited in the same year (Ingold 1952), when *Actinospora megalospora* was described: «a rich collection of spores belonging to known species (of aquatic Hyphomycetes)» concentrate in «scum, often foamy,...gathering here and there behind twig-barriers on the surface of a stream». It is interesting that even as late as 1953, Ranzoni reported an extensive search of aquatic hyphomycetes in California without ever mentioning foam. Had he been made aware of this source, his species list would certainly have been much larger.

Going back to Ingold's original paper, after examining «many water samples», 20 different kinds were illustrated; four could not be isolated (the then recently discovered antibiotics were obviously not readily available for fungal culture during World War II), but the remaining 16 were shown to be conidia of fungi which he formally described in culture as hyphomycetes (see updated list in our Table 1).

Table 1
The «aquatic Hyphomycetes» of Ingold (1942)

Alatospora acuminata gen. sp. nov.	*Lunulospora curvula gen. sp. nov.*
Anguillospora gen. nov.	*Margaritispora aquatica gen. sp. nov.*
**Anguillospora longissima comb. nov.*	*Tetrachaetum elegans gen. sp. nov.*
**Articulospora tetracladia gen. sp. nov.*	*Tetracladium marchalianum*
**Clavariopsis aquatica*	*Tetracladium setigerum comb. nov.*
Flagellospora curvula gen. sp. nov	**Tricladium splendens gen. sp. nov.*
Clavatospora (as Heliscus) longibrachiata sp. nov	*Tricladium angulatum*
**Heliscus lugdunensis(as H. aquaticus)*	*Varicosporium sp. nov. elodeae*
Lemonniera aquatica	

(* known to have a teleomorph).

Sporulation on *Alnus* leaves was discovered four months after conidia had been first encountered, and was believed to occur underwater. Nine species were new to science. Seven species were placed in new genera on the basis of not only the morphology and ontogeny of the conidia and conidiogenous cells (which had already been granted taxonomic value by Vuillemin, Mason and other pioneers of modern anamorph taxonomy) but also on the arrangement, numbers and form of conidial branches, which were seen to be stable characters within species. A physiological

criterion, aquatic sporulation, was also introduced in the characterization of his fungi, and «aquatic spores», in contrast to Mason's «dry» and «slime spores», were defined as those «produced, liberated and normally dispersed below water». The term «aquatic Hyphomycetes» was proposed for this presumed new biological group of fungi. Ingold had thus reached the conclusion that, although these fungi were a polyphyletic group, they had probably adapted from a terrestrial existence to one in flowing waters, and even theorized on evolutionary lines developed in freshwater habitats. Nilsson (1964b) further speculated that aquatic hyphomycetes may have evolved towards the production of simplified, well-developed, tetraradiate or sigmoid conidia, as these were apparently the most common and widespread spore types, and to be totally submerged in running waters. The term «aquatic Hyphomycetes» was generally accepted at least until Price & Talbot (1966) first reported stauroconidial hyphomycetes from dry streambeds in Australia.

However, when the term «aquatic hyphomycetes» is used, we should be aware of the following qualifications:

A) The representative anamorph is not always aquatic

— The difference between submerged and surface sporulation was first noticed by Petersen (1961), who thus referred to aerospores and aquaspores within the same anamorph.
— From pure culture observations in standing water, a number of species with aquatic sporulation form conidia at the interface, but not underwater. Whether this feature is maintained in flowing water or in the presence of nutrients is not known.

B) Other parts of the life cycle may be out of water too:

— Aquatic synanamorphs are known for a number of «aquatic Hyphomycetes», but some are only produced aerially, such as the arthroconidia of *Clavatospora longibrachiata* (Ingold & Cox 1957). In *Filosporella versimorpha* (Marvanová *et al*. 1992) the representative morph is produced in water but the phialidic microconidial morph may be formed aerially or underwater; and arthroconidia are said to be produced only on unsubmerged cultures.
— Since Ranzoni (1956) found that *Flagellospora penicillioides* was really an anamorph of a *nectriaceous ascomycete*, a growing number of «aquatic hyphomycetes» (including six among those originally studied by Ingold; see asterisks in Table 1) are being shown to bear teleomorphs among the asco- or basidiomycetes. Except for a few species such as *Loramyces juncicola*, they are produced at the surface of or outside water, albeit under moist conditions. From observations for example of easily recognizable lep-

tosphaeriaceous ascospores in water and foam samples, it is most probable that those of *Massarina* can also be dispersed on or under water, but their source is presumed aerial. Aquatic dispersal may also be expected of the less recognizable aerially producedd ascospores of the Leotiales as well as of basidiospores. The term «amphibious fungi» has been proposed as an alternative for aquatic hyphomycetes, but it should exclude the numerous species of so far anamorphic fungi believed to produce their conidia underwater, and thus capable of leading a completely aquatic existence.

— In at least some species, microconidia produced underwater in all probability have a spermatial function. Therefore sexual reproduction, although occurring in most species aerially, would partly depend on aquatic dispersal. It is not known if aerial synanamorphs are involved in spermatization. The functional interplay and biological significance of all these morphs is still a mystery.

C) Some species probably complete their life cycles outside water

Bandoni (1972) and several other authors have observed litter colonization by «aquatic hyphomycetes» far from permanent or temporary waters (see below). *In situ* asexual sporulation has sometimes been proven, but it is not clear whether conidia are produced aerially, superficially or even possibly underwater. In laboratory incubation, forest litter has yielded conidia either underwater (Webster 1977) or after moist incubation.

D) Other fungi may need contact with free water for sporulation

— There are hundreds of poorly studied soil-and litter fungi with stauro- or scolecoconidia seldom found in streams. There is a need clarify if they or their supporting structures require contact with free water for development. If so, these species would at least form a continuum with the aquatic Hyphomycetes.

— «Geofungi» with little differentiated conidia are frequent in streams (see below) and their sporulation requirements need to be studied.

Ingold himself (1979) implicitly expressed doubts on the correctness of the term by referring to his fungi as the «so-called aquatic Hyphomycetes».

In our biodiversity studies in terrestrial waters initiated in the seventies we have been largely isolating conidia from foam samples and thus of unknown source. We first assumed that they would be true aquatic hyphomycetes, but due to their highly varied water requirements and to the gradual discoveries of their teleomorphs, we soon realized the need for a term devoid of any taxonomic, physiological or ecological connotations. The term «Ingoldian fungi» was thus considered appropiate (Descals *et al*. 1977), as it would at the same time honour Professor In-

gold's achievements in the field. But the term is provisional until such time as we can specify criteria that will properly define what is generally believed to be a distinct biological group.

At the moment, the Ingoldian fungi could be defined as a loose assortment of filamentous fungi typically adapted to asexual sporulation (or to the production of at least the macroconidial anamorph if others are present) in relatively undisturbed lotic systems. It is here where these conidia are found most frequently, although some are also present in lentic waters and even outside water. Asexual sporulation in most species studied responds to contact with free water and turbulence, as well as to low dissolved nutrient levels. Depending on the species, conidia may be formed underwater, at the air-water interface and/or aerially. They mostly have a high surface/volume ratio (which could be advantageous for assimilation in nutrient-poor environments or for dispersal on the surface of water), and are typically large (which should aid in their being trapped onto substrates). Stauroconidia (i.e. those with more than one element) can be ontogenically tetraradiate (i.e. with four arms originating from a common point), but they are much more commonly «tricladioid»: with an axis supporting two or more lateral branches at different levels (Descals 1985). More complicated shapes also occur. Irrespective of the origin of branches, a feature of most stauroconidia is that they have several ends (i.e. apices and basal detachment point), which is from where germination and substratum colonization are commonly initiated. Probably also important is the fact that these apices are in more than one plane, thus probably increasng chances for contact and attachment when tumbling in turbulent waters. Scolecoconidia, on the other hand, with typically two contact points (Webster 1987), are probably less adapted to stream conditions and thus less common and diversified here. It is not clear if germination proceeds here as easily from intercalary cells or detachment points. At least in some species, mucilage (extruded while in suspension and/or upon germination) and appressoria further aid in anchorage and colony establishment in turbulent waters (Au *et al*. 1996), (although neither of these two features need be only attributed to the Ingoldian fungi). Mostly non-submerged ascomatal and basidiomatal teleomorphs lacking any morphological adaptations to water are present in a number of species. The ascomycetes often have aquatic or sometimes aerial synanamorphs.

It should be pointed out that, although many Ingoldian fungi are convenient for ecological studies because their species-diagnostic conidia obviate the need for pure culture, 1: not all species may be important in decomposition, some not even being necessarily saprotrophic (e.g. *Crucella subtilis*, see Marvanová & Suberkropp 1990); and 2: there is no evidence that non-Ingoldian fungi growing in mixed submerged communities cannot be similarly active in decomposition.

b) «Facultative aquatic hyphomycetes»

Some of the staurosporous dematiaceous forms initially seen by Ingold, and later compiled in his identification guide of 1975, were considered by him as ecolo-

gically distinct from the typical «aquatic hyphomycetes». Goh & Hyde (1996) have recently proposed that such fungi constitute a distinct biological group. This would include such genera as: *Camposporidium, Canalisporium, Casaresia, Diplocladiella, Nawawia, Setosynnema, Tetraploa, Triscelosporium* and *Pleiochaeta*. It is said to be made up of hyphomycetes (some with known teleomorphs) with mostly macronematous conidiophores (though not so for example *Casaresia*) and thick-walled, often branched conidia. None are scolecosporous. Mycelia typically colonize wood. Conidia are sometimes found in water, but sporulation is normally aerial or possibly also interfacial (although *Casaresia sphagnorum* and *Diplocladiella scalaroides* can sporulate underwater *in vitro* and are rather frequent in streams). The group is being called the «submerged aquatic hyphomycetes», or also the «facultative aquatic hyphomycetes» (*sensu* Thomas in Goh & Hyde 1996). It is possible that this group forms an ecological continuum with the terrestrial dematiaceous hyphomycetes.

c) «Terrestrial aquatic fungi»

Tubaki (1957) and co-workers (Ando & Tubaki 1984 a,b,c), who had been studying fungi in freshwater habitats, described a group of hyphomycetes with stauroconidia strikingly similar to those of the Ingoldian fungi, but which they collected in water dripping off trees. Tubaki *et al*. (1985) later associated the presence of fungi such as *Trinacrium* and *Tripospermum* with dew formation on *Miscanthus, Quercus* and *Magnolia* trees. Sporulation was enhanced when drops of water were placed on agar cultures. Those of *Tripospermum acerinum*, for example, produced roughly twice as many conidia in drops placed on the colony margins as in controls.

In 1989, Ando & Kawamoto defined a new biological group which they termed, the «terrestrial aquatic» fungi, as an assemblage of mostly moniliaceous hyphomycetes with micronematous conidiophores and branched conidia which seem to have evolved on trees rather than in streams. (It is curious that scolecoconidial morphs have not yet been reported here). Sporulation is «stimulated with water». These authors believed that conidia are designed to «hold water around them as long as possible». It is not clear if they tried submersion for sporulation. Genera such as *Alatosessilispora, Arborispora, Curucispora, Microstella, Ordus, Retiarius, Titaea, Tricladiella, Trifurcospora* and *Trisulcosporium* were included, but new species were also described in anamorph genera with representatives often found in streams, at least as conidia; e.g. *Dicranidion, Dwayaangam, Trinacrium* and *Tripospermum*. Branched spores of undescribed fungi (some known to be of a conidial nature) have later been observed by us in water shaken off tree leaves in the misty laurisilva of the Canary Islands in the Atlantic, as well as from graminaceous bushes (of *Chusquea*) in humid forests in the Argentinian Patagonia (Descals *ined*.).

The growth habits of the «terrestrial aquatic» fungi on live terrestrial plants are not known. They may colonize live tissues as endo- or epibionts respectively, or in senescent or dead tissues, or even on arthropods or other fungi. *In situ* sporulation

requirements are even less known. The response to dew formation should certainly be studied.

Through observations made with a continuous perfusion technique described by us (see below), it was seen that if water was dripped off a sporulating colony growing in a film of water, conidia would mostly remain behind, probably due to surface tension. This may also happen with dew drops in nature, and its possible biological relevance could be worth checking. On the contrary, rain or dew drops impacting on the sporulating colonies would help dislodge the conidia and aid in dispersal by splashing them away.

The term «terrestrial Ingoldian hyphomycetes» was later proposed by Ando & Kawamoto (1990). Although either this name or that of «terrestrial aquatic fungi» may not be accepted by everyone, the preferred ecological niche of this group of fungi is quite distinct from that of the Ingoldian fungi.

An interesting borderline case is that of *Tetracladium maxilliforme,* included in a typically Ingoldian genus. It was first recorded by Rostrup (1894) on sclerotia *of Typhula trifolii* parasitizing *Trifolium pratense*. Whether conidia had been produced in moist air, in/on dewdrops or otherwise was not mentioned. There are several other records of this mycophilous fungus on terrestrial plants. Sporulation, however, is known to occur underwater *in vitro*, and although this species is infrequent as conidia in most streams studied, it has been reported to colonize submerged leaf baits in streams (e.g. Bärlocher & Schweizer 1983).

Another curious case is that of the basidiomycete *Titaeella capnophila*, described by Arnaud (1952) as a parasite on sooty moulds. Ando & Tubaki (1985) redescribed it from conidia in rain drops fallen from trees. It has been recently isolated as a conidium in stream foam by Descals (1997). The species, however, does not qualify as a typically aquatic resident.

d) «Geofungi», or terrestrial aerially-sporulating fungi in streams

Anamorphs with conidia showing little differentiation frequently appear on submerged substrates after plating or dilution techniques. Tubaki (1957) initially observed that some of these could sporulate in shallow water. Nilsson (1964a, p. 11) later stated that «many terrestrial fungi will grow and sporulate in water... Sporulation will often occur both in or on the surface of water; also, rarely, below». Price & Talbot (1966) listed species of *Phialocephala, Stachybotrys, Alternaria, Moeszia* and *Acremonium* (as *Cephalosporium*) on leaves collected from water.

Evidence of underwater colonization was provided by Kaushik & Hynes (1971): leaf baits submerged in a stream for up to seven months and «dilution-plated» on Potato Dextrose Agar (PDA) yielded hyphomycetes in the genera *Alternaria, Cladosporium, Epicoccum, Fusarium, Penicillium* and *Trichoderma* as well as other fungal groups (the Coelomycetes *Phoma* and *Coniothyrium,* yeasts tentatively placed in *Candida* and unidentified Mucorales). These so-called geofungi colonized the leaves within one to two weeks' submersion, and mycelia were present in significant

quantities during most of the seven months' submergence; they should thus be considered aquatic residents although their role in decomposition has not yet been studied. Bärlocher & Kendrick (1974) submerged pre-sterilized *Acer* leaves in a stream for four weeks, and isolated two species of Mucorales, several fusaria and penicillia, 24 other aerially sporulating hyphomycetes, five coelomycetes and an *Acaulopage* (Zygomycetes), plus some generally recognized aquatic residents, i.e. two species of aero-aquatic hyphomycetes, two oomycetes and 19 species of «aquatic hyphomycetes». Chamier *et al.* (1984) observed that *Cladosporium* and *Epicoccum* isolates from *Alnus* leaf baits were found to «degrade substrates representative of cell-wall polymers vigorously whereas aquatic hyphomycetes showed varied degradative ability». These and other soil or litter fungi were present in her stream as live mycelia throughout eight weeks of submersion.

Submerged decaying macrophytes such as *Ranunculus* can also be colonized by presumed soil and litter fungi such as *Trichoderma, Rhizoctonia, Tolypocladium, Fusarium tabacinum* and *Sympodiophora,* as proven by Kirby (1987) with particle-plating and washing techniques.

Tree and fern roots growing underwater in streams have also yielded species of *Aspergillus, Cladosporium* and *Penicillium* (apart from the coelomycete *Pestalotiopsis* and Zygomycetes in *Mucor and Rhizopus*) by particle plating (Raviraja *et al.* 1996). An endophytic habit was suggested. We do not know the source of the inoculum for root colonization, but the authors reasoned that it must have come from water because the outer bark was more heavily colonized than the wood underneath. *In situ* sporulation was not checked for but could have occurred on the outer, presumably dead tissues. Internal mycelia would probably have to await exposure to air or water through substrate decomposition before sporulating. There is also the possibility that live wood chemically represses sporulation.

e) Aero-aquatic fungi

These are typically found in lentic, often stagnant waters, although substrates in or near streams are also colonized. Teleomorphs, when present, are produced out of water. The representative anamorph is produced in air or at the air-water interface, with propagules relatively large and morphologically adapted for trapping air and floating, i.e. they are helicoid in two or three dimensions, or densely and shortly branched, or in some species made up of anastomosing or loosely intertwined hyphae (which also trap air), or even of pseudoparenchyma. The aero-aquatic fungi were a quite well defined biological group until *Spirosphaera dimorpha* was recently described by Marvanová & Bärlocher (1998). This is a remarkable fungus with one morph bearing air-trapping propagules and another one producing the stauroconidial aquatic *Lambdasporium*. This opens up the possibility of finding further species with dual strategies for asexual reproduction and dispersal in both static and running waters. Techniques enabling contact with free water should be added as a routine in future descriptive work of the aero-aquatic fungi.

FUNGAL PROPERTIES AND ENVIRONMENTAL CONDITIONS RELATED TO CONIDIAL PRODUCTION

Fungi such as the Laboulbeniales, basidiomycetes and Teliomycetes, many coelomycetes, and most parasitic, lichenized or mycorrhizal fungi can or have to be described exclusively from reproductive organs produced in the field. In contrast, many saprotrophic filamentous fungi can be cultured and produce their anamorphs on agar media. However, most Ingoldian fungi which belong here need contact with free water for sporulation. This is one of their most characteristic features, but it is unfortunately not well understood. Under submerged conditions, and one dare say even in thin water films, water is the medium in which these fungi breathe, feed, excrete and reproduce asexually. It should be remarked that in pure culture, immediately upon coming in contact with the fungal colony and its substratum, previously distilled water changes (sometimes drastically) its physiochemical properties as a result of diffusion and fungal wall leakage, and sporulation thus really takes place in weak aquatic solutions rather than in pure water. This may be extrapolated to natural situations.

Some Ingoldian species, probably a minority, sporulate readily if we partly submerge a piece of the colony in distilled water. Others require different conditions (e.g. water aeration, agitation and water or nutrient renewal) which have all too often been applied empirically and cannot yet be explained. Thirdly, there are always a number of isolates which either do not sporulate under the above conditions or do so very poorly, thus remaining undescribed. We therefore felt the need to have a better understanding of two basic aspects related to exposure to water: 1- fungal properties affecting asexual reproduction; and 2- the effect of environmental (physiochemical) conditions on conidial production. Over 1500 publications now exist on the Ingoldian fungi, but the above aspects have not yet been reviewed. Such an undertaking requires a perspective which only a reproductive physiologist can have. Due to this, and also to lack of space, we have instead selected some of the more relevant observations and experiments, and arranged them into a scheme which we hope will help analyze the problems in future research.

A) Some fungal properties related to asexual reproduction

A1. The fungal individual

A1a. Vegetative growth vs. sporulation

Sporulation levels on leaves can now be related to the total mycelial biomass, as this is estimated by ergosterol extraction (Gessner *et al*. 1991). We also know that conidial production can occur during vegetative growth (see sporulation curves in e.g. Suberkropp 1991and Gessner & Schwörbel 1991). But colonies grow on leaves intermixed with those of other species (and possibly of the same too, although intraspecific relationships have been little studied. Until new (e.g. serological, ra-

dioactive or now molecular) labelling or strain identification techniques are refined, it is impossible to determine mycelial biomass for any one species and relate it to its levels of sporulation. In pure culture, quantification is possible if the mycelia are grown in liquid media. (Alternatively, they may be separated from special gelified substrates). But sporulation levels are also here meaningless unless related to the amounts of mycelial biomass present at that moment, because the mycelium keeps growing while it sporulates. Vegetative growth can occur at very high rates in the presence of turbulence; apart from the fact that an unknown but probably significant portion of this biomass is reproductive, i.e. made up of conidiophores and developing conidia, and that this proportion may change with species, age and environmental conditions.

A1b. On the reproductive maturity of mycelia

In streams, there is often only a short delay in conidial production after leaf baits are colonized. *Lunulospora curvula* and *Clavatospora tentacula* produced conidia after only seven days (Suberkropp & Klug 1974, fig. 2), and Canhoto & Graça (1996) reported sporulation of three species after only three days. This is presumably the time needed for the first inocula to establish reproductively mature colonies. Ingold (1942) first saw that colonies of all but two of the 13 species he cultured sporulated at a very early stage; and Webster (1959) and Nawawi (various papers), among others, have observed *in vitro* sporulation in some species after only one week's colony growth.

In the other two species cultured by Ingold (1942), *Margaritispora aquatica* and *Heliscus lugdunensis*, conidia were only produced on old parts of colonies after submersion, which suggests that an inhibitor had to be degraded. Nawawi (1974b) later discovered that *Dendrosporomyces splendens*, a conidial basidiomycete, did not sporulate until after five to six weeks' growth. There is practically no information for other species. If this presumed delay in the attainment of reproductive maturity of hyphae in some species is not an artifact of culture, delays and even species successions in the field could be caused by species needing different times for reaching reproductive maturity, and may not only be due to competition and antagonism.

An interesting, yet unexplained, response was observed by Gönczöl *et al.* (1990): young cultures of *Vargamyces aquaticus* required submersion in shallow water for sporulation, while four- to five-week old cultures sporulated even without submersion. In this case, it could be that a soluble inhibitory factor needs to be overcome or broken down for sporulation to occur.

A1c. Staling (i.e. loss of sporulating ability) of colonies *in vitro*

In industrial systems with continuous culture, factors controlling sporulation

have been intensively studied, and this can in some cases be manipulated at will. But not much is known on the sporulation behaviour of many Ingoldian fungi. On agar media, *Alatospora acuminata* (Ingold 1942) and *Lemonniera terrestris* (Ingold 1958, as *L. brachycladia*) are claimed to become sterile after a few weeks' growth *in vitro*. Ranzoni (1953) and later Umphlett (1957) also observed that old cultures of *Lunulospora curvula* gradually lost the ability to sporulate. The latter author believed that this loss might be irreversible. However, Marvanová & Bärlocher (1988) found that *Taeniospora nasifera* recovered its sporulating ability after subculturing from the growing edge of a colony which had become sterile after the first transfer.

More research is needed to establish wether staling is of an environmental and/or genetic nature. On leaf baits in streams, although substratum availability presumably need not be limited, Ingoldian fungi seem to cease sporulating after varying periods; this could be due to ageing, but in a field situation antagonistic and/or competitive relationships with other species or with different colonies of the same species may also intervene. It is not known either if these mycelia actually die or are killed, or just rendered sterile.

A1d. Restricted growth

As happens with other groups, when isolating Ingoldian fungi a number of unidentified cultures (which are not believed to be contaminants) show «restricted growth»; i.e. growth is very slow and mostly halts after the colony has reached a few mm diam. have been reached. Standard techniques, whether submerged or not, fail in inducing sporulation, and identification by morphological methods is thus impossible. Nawawi & Kuthubutheen (1988) reported restricted growth for three isolates presumed to be of *Condylospora* species, and we have regularly observed the same response through many years of pure culture of other fungi. We do not know if this is a species character or if it affects only some isolates. The cause could also be environmental or pathological. In the conidial basidiomycete *Naiadella fluitans,* a colony that had grown only two to three mm in diam. after 30 days on MA and MYP yielded abundant conidia when put in contact with water (Marvanová & Bandoni 1987).

A1e. «Submerged sporulation»

Colonies of some fungi, either underwater or not, may sporulate inside the agar medium. In terrestrial hyphomycetes (e.g. *Paecilomyces* spp., in Okada *et al.* 1995) this is sometimes called «submerged sporulation» (a confusing term in the context of aquatic fungi, where submersion normally implies inmersion into water). This response may have to do partly with the water activity of the agar. Hudson (1961) first observed «submerged sporulation» among the Ingoldian fungi when

growing *Heliscus submersus* on 2% MA. Webster & Descals (1975) reported the same for *Casaresia sphagnorum*, also on 2% MA.

Pseudothecia of leptosphaeriaceous ascomycetes from coastal lagoons have been seen by us to form within agar, although here it may be a thigmotactic response to solid substrates, as they are firmly adhered to the base of the plates.

A1f. Sporulation at the infraspecific level

Physiological requirements of asexual reproduction in the Ingoldian fungi have rarely been considered below the species level, although there are morphotypes, forms and varieties recognized within species of several genera (e.g. *Alatospora, Articulospora, Taeniospora* (Marvanová & Descals 1985). These may be really genotipically separated ecotypes.

Three fungal properties may affect conidial counts in studies on sporulation. Two are related with conidial characters, and the third one with sporulation dynamics:

A1g. Conidial stickiness

Petersen (1961) first observed *in vitro* mucilage production at conidial tips in *Anguillospora longissima*. This has since been seen in pure cultures of a number of species as well as in stream foam. Mucilage production is claimed to be a thigmotactic response (Au *et al*. 1996), but detached conidia may be sticky even when in suspension. This is easily seen when handling conidia with mounted hairs or Pasteur pipettes for isolation. It is not known if stickiness varies between conidia of the same or different species, as, in increase with age, but it does suspensions such as liquefied foam, conidia will adhere to each other after several hours and then settle. (As a matter of fact, this entanglement should theoretically lead to more efficient trapping, but it is not seen in freshly collected foam, even if old, nor on membrane filters). Conidia also adhere to solid surfaces and are thus difficult to sample. In standing water, they settle onto the bottom of dishes and become so firmly attached that they cannot be resuspended without damage. In aerated flasks, conidia are often kept in suspension for up to several days, and a number may thus adhere to the inner walls. It is not known if in larger vessels and at lower temperatures adherence is less serious. Smearing the inside walls of the sporulation chambers with oil, as is done in bacteriology (J. Lalucat, pers. comm.), could solve the problem. Potassium hydroxide (KOH) at low concentrations has been seen by us to neutralize the adhesiveness of conidial mucilages. But oil, and certainly KOH, may affect conidial viability. However, this may not be a problem if isolations are not needed. There is definitely a need to determine the importance of conidial adhesion to container walls in the various sporulation techniques compiled below.

A1h. Conidial buoyancy

Tubaki (1957) and later authors remarked that conidia of some Ingoldian fungi either floated indefinitely or eventually sank. (Those of *Varicosporium elodeae* were not wettable and floated, even though they were produced underwater). When sampling conidial suspensions by pipetting, surface conidia will be left behind as water is being sampled from below. If done by decanting, the surface scum bearing the conidia will glide in the opposite direction to the flow and remain behind (Bandoni 1972) and on the walls of the container. Wetting agents should thus be tested for sinking conidial suspensions prior to sampling.

A1i. Microcycle conidiation, production of secondary conidia and conidial fragmentation

These three phenomena, seen in cultures of a number of species, may cause an artificial proliferation of propagules and significantly alter results in sporulation studies, especially if the liquid medium is kept unchanged or if incubation is for longer periods. Lowe (1927) first observed that conidia of a fungus closely related to *Cerasterias raphidioides* var. *inaequale* (probably *Tetracladium setigerum*) produced a new generation within 48 h. Ingold (1942 Text fig. 21) illustrated microcycle conidiation in *Tetracladium marchalianum*, and Price (1964 fig. 9) saw germination by repetition in a species of *Tricladium*. *Articulospora tetracladia* is possibly the best known case of secondary sporulation, first seen by Marvanová & Marvan (1963) and further studied by Khan (1986). Secondary conidia tend to be smaller and perhaps less branched, but they may not be easily distinguished from primary conidia. There is an exception in *Gyoerffyella rotula*, where the lower primary branch of secondary conidia arises from the lower cell on the axis, while in primary conidia it sits on the second cell (Marvanová, 1975). Conidial fragmentation is common for example in species of *Dendrospora,* among others. It must be pointed out that in such species as *Varicosporium elodeae* (Lindau, 1910, Ingold 1942), *V. giganteum* (E. Chauvet, pers. comm), *Arbusculina fragmentans* (Marvanová 1988) and *Pseudozyma prolifica* (Bandoni 1985), etc., where conidial elements are all alike, conidia are not always distinguishable from part-conidia, and this may present problems when counting.

It is not known how the above phenomena respond to environmental conditions such as temperature and water or nutrient renewal, nor is there any evidence of their occurrence in nature.

A2. Inter-relationships with the stream community

Relationships between Ingoldian fungi and macroinvertebrates have been studied in the context of litter decomposition (although there is no information on the effects

of shredders on *in situ* sporulation), and will not be discussed here. A better understanding is needed, however, of interactions with other microorganisms. A few studies have been initiated in the last decade with laboratory micro- or mesocosms (see below), which should be useful tools for future experimentation. As applies to *in vitro* species interactions among the Ingoldian fungi, maybe the most striking and still unexplained example is that reported by Webster *et al.* (1976), who observed that when *Tricladium chaetocladium* and *Lunulospora curvula* were grown in mixed culture the optimum sporulation temperature dropped from 20 to 5 °C and from 25 to 10 °C respectively. Mixed growth in leaves may thus at least partly explain why *T. chaetocladium* is typically a winter-sporulating species in cold-temperate climates. It does not however explain why *L. curvula* is predominantly a summer-sporulating species there. (Note that conidial counts in this study were performed after two days' aerated submersion in separate cultures, but after seven days in mixed cultures, which seems exceedingly long; conidia may have adhered to the walls of the vessel, especially at the higher temperatures, and that could partly explain the lower counts). Interactions between other Ingoldian species have not been tested, nor do we know what mechanism operates in such drastic changes in sporulation requirements.

Results on antagonism between Ingoldian fungi in streams are ambiguous. There is no information on wood colonization, but, as stated above, different mycelia can grow intermeshed on the same leaf. Shearer & Lane (1983), in a detailed study recording sporulation on a stream-colonized *Acer* leaf cut into 6 × 6 mm squares, found at least seven species of Ingoldian fungi sporulating together, but only after laboratory submersion.

Other reports are also on vegetative growth parameters. Chamier *et al.* (1984), when plating 2 × 2 mm squares of *Alnus* leaves on a nutrient medium, proved frequent colony overlap by plating techniques. Gulis & Stephanovich (1999) stated that, of 29 species studied in dual culture, only four demonstrated antagonistic activity on the vegetative growth of the other fungi. On the other hand, Shearer & Zare-Maivan (1988) paired a number of species on agar media and observed that in 97% of the interactions one or both members inhibited the growth of the other. Possibly diffusible antifungal substances were produced in some cases, as zones of inhibition were apparent. Furthermore, there were no stimulation reactions between any pairings. Fisher & Anson (1983) have also observed an inhibition of mycelial growth on wood blocks and of conidial germination of various Ingoldian species by *Massarina aquatica (*anam. *Tumularia aquatica).* There is as yet no information on the effects of species interactions on sporulation. Submerging all the above cultures could have ready provided invaluable information on interaction effects on sporulation *per se*.

With regard to interactions between Ingoldian and other fungi, little is known, especially on *in situ* field sporulation. Chamier *et al.* (1984) suggested inhibition of other hyphomycetes such as *Cladosporium* and *Epicoccum* by some Ingoldian fungi, but it was concerned with vegetative growth. Interactions with oomycetes deserve further study. On laboratory-incubated leaves Tubaki (1957) first observed that oomycetes (as "Phycomycetes"), aero-aquatic fungi and ascomycetes fruited only after the Ingoldian fungi had sporulated. Nilsson (1964a) further stated that In-

goldian fungi were rare where «Phycomycetes» abounded, most probably also making reference to *in vitro* sporulation on leaves after submerged incubation.

In the case of microorganisms other than fungi, early workers such as de Wildeman (1893) believed that bacterial contaminants interfered with the vegetative growth (and presumably sporulation) of fungi such as *Tetracladium* (possibly *T. marchalianum*). Ciferri (1959) attributed the cessation of sporulation on skeletonized leaves in standing, non-sterile water to the presence of either bacteria, algae or protozoa. Sporulation was resumed after replacing the water, but this could have also caused a dilution of soluble nutrients or other staling compounds. Sridhar & Kaveriappa (1984) observed sporulation leaf pieces colonized for 60 days, replacing water every other day for controlling bacterial levels. On the contrary, Roldán (1991) observed heavy sporulation of colony pieces of *Ypsilina graminea* (as *Volucrispora graminea*) if submerged under non-axenic conditions, but not so in standing sterile distilled water. (Other isolates of this species, however, have been seen by us to sporulate readily in pure culture).

Nawawi (1974c) reported that *Tricladiomyces malaysianus* sporulated abundantly in the presence of contaminant bacteria (even without submersion), and he later (Nawawi, 1976b) recorded sporulation of *Condylospora spumigena* on various media only when bacteria were present. Bengtsson (1992), working with continuously renewed aerated water, observed that total conidial production of a community of six Ingoldian species was higher in the presence of certain species of bacteria. *Articulospora tetracladia*, however, sporulated less under these conditions.

The presence of an antibacterial antibiotic-producing fungus on a leaf would presumably benefit the survival and possibly the sporulation dynamics of a non-antibiotic producing species sharing that particular habitat, but no data on this are avalaible.

In the search for antibiotics, the reverse phenomenon, fungal antagonism to bacteria, has been intensively studied. Fisher & Anson (1983) first reported a slight inhibitory effect of *Massarina aquatica* (anam. *Tumularia aquatica*) on *Bacillus* or *Staphylococcus* in dual culture. Later, Harrigan *et al*. (1995) discovered that anguillosporal, a benzaldehyde derivative produced by *Anguillospora longissima*, and also with a *Massarina* teleomorph, had antibacterial properties. Platas *et al. (*1995) recorded 14 species of Ingoldian fungi with activity against *Bacillus subtilis*, and Gulis & Stephanovich (1999) found bacterial inhibition by 15 species of Ingoldian fungi.

Filamentous bacteria (i.e. actinomycetes) can be conspicuous on leaves decaying in fresh water (Tubaki 1957, Willoughby 1978), but their effects on either growth or sporulation of Ingoldian fungi have not been explored.

What little information there is on viruses, algae, protozoa and nematodes shows negative effects of these on fungal growth. There is one contrasting reference on the effects on rotifers. Barron (1991), found that *Dwayaangam heterospora*, a weak parasite of rotifer and nematode eggs in soils producing spores only aerially but classified in an anamorph genus containing Ingoldian fungi, was seen to sporulate (aerially) much better in the presence of rotifers under near UV light. (The possible independent effect of this radiation was not considered).

B) Some environmental conditions: water relations

B1. Ecological conditions

B1a. Outside large water bodies

The sporulation of Ingoldian fungi in non-submerged habitats can occur in basically three micro-environments. The conidia of some species can undoubtedly be produced aerially (in moist air or possibly also in condensation drops), at least in culture (e.g. *Lemonniera terrestris*). But interface sporulation, i.e. in/on water films on wet substrata, is probably more common. Surface-active substances at the air-water interface and immediately below create what are called «microlayers», where proteins and lipids are believed to be a rich nutrient base for the neuston. Microlayers may contain up to five times as many microorganisms as in the water column beneath them (Bandoni 1974). They can also form on or between surfaces in terrestrial litter, on temporarily exposed wet substrates along stream banks and lake shores, on living plants and in the water fraction of soil. At least some stauroconidia found in such microlayers are of Ingoldian fungi. These could therefore be a medium for their sporulation and dispersal in terrestrial situations.

In culture, Lindau (1910) first observed interface sporulation in *Varicosporium elodeae* on conidiophores growing from beneath the water surface. According to Karling (1935), *Tetracladium setigerum* (misidentified as *T. marchalianum)*, when grown in liquid media, sporulated only at the interface. Ingold (1942) observed how submerged conidiophores or conidiogenous cells of *Heliscus lugdunensis (*as *H. aquaticus), Margaritispora aquatica* and *Varicosporium elodeae* pierced the water surface to release their conidia above it. Descals & Webster (1982) made similar observations on *Tricladium terrestre*.

Thirdly, sporulation may also take place under temporarily submerged conditions (e.g. vernal pools resulting from snowmelt, rain pools, tree holes, etc., see below).

Three habitats are known to be exploited outside streams by some Ingoldian fungi: live terrestrial plants (where conditions may however be generally too dry), forest litter (where moisture is higher and more constant) and soil (a still unexplored habitat).

B1a1. Live terrestrial plants

So far, a few species of Ingoldian fungi have been found, always in low amounts, associated with underground or aerial organs of live terrestrial plants, where they may exist as saprotrophs, endobionts or parasites. There is evidence that sporulation may occur on live organisms (plants or animals), but in other cases the invaded tissue must first die (e.g. resulting in necrotic spots on leaves).

a) Aerial parts

Mycocentrospora acerina, which has received much attention due to its pathogenicity on commercially grown crops, was probably the first Ingoldian fungus recorded outside streams. Von Thümen (1876, *in* Hartig 1880) first obtained cultures from necrotic spots on *Acer* cotyledons and leaves. Much later, Sprague (1937 in Newhall 1946) saw conidia on leafspots of *Osmorhiza brevipes* (*Apiaceae*) along stream banks, Neergaard & Newhall (1951) collected them in water drops placed on live *Apium* stalks, and Viennot-Bourgin (1955) observed profuse sporulation in water drops on lesions of *Viola* leaves after moist incubation.

Records exist of two other pathogens on aerial parts: *Gyoerffyella entombryoides,* seen on necrotic spots on dying stems of *Rosa* (Boerema & von Arx 1964), and *Tetracladium cf. marchalianum*, on diseased sheaths of *Allium porrum* (Puttemans, *in* de Wildeman 1920).

Apart from the conidia of «terrestrial aquatic fungi» found in waterdrops falling from trees (discussed above), Tubaki *et al*. (1985) also collected those of *Articulospora tetracladia*. This species may however be a complex of two biotypes occupying different niches, one of them with sporulation less dependent on submersion, and thus being better adapted to a terrestrial existence. Mycelia probably grew either saprotrophically or in live tissues.

b) In underground organs

Through plating techniques preceded by surface sterilization, it is known that a few Ingoldian species inhabit live underground plant organs. Some are on herbaceous plants. For example, *Varicosporium elodeae* has been recorded from inside roots of *Phaseolus vulgaris* (Taylor & Parkinson 1965) and from cold-stored, apparently healthy *Fragaria* crowns and roots (Gourley 1969), and *Tetracladium marchalianum* from pieces of *Fragaria* root tips (Nemec 1970). Both these crops are often irrigated, the habitat thus in this case not being fully terrestrial. *Tetracladium cf. marchalianum* has also been seen attacking *Hyacinthus* (*Liliaceae*) bulbs (Sorauer in de Wildeman 1920). The diagnostic technique was not specified, but it probably was through conidial detection after moist incubation. Perennial plants are also colonized by at least one Ingoldian fungus. Fisher & Petrini (1990) obtained *Tricladium splendens* from several surface-sterilized bark pieces taken from live roots of *Alnus glutinosa* growing outside a leat (presumably a moist but not submerged habitat). It was not stated whether the fungus colonized or sporulated on living or dead tissues.

c) On roots

Neergaard & Newhall (1951) collected conidia of *Mycocentrospora acerina* in water drops placed on infected *Primula* roots; and those of *Varicosporium elode-*

ae were recorded on healthy *Fagus* seedling roots by Waid (1954) by plating techniques without previous surface sterilization. *V. elodeae* was later isolated (albeit infrequently) by plate dilution techniques, also without surface sterilization, from the roots of *Phaseolus vulgaris* (Taylor & Parkinson 1965, Parkinson & Thomas 1969). Nothing is known of the growth habit or *in situ* sporulation conditions in these fungi. They could exist as endobionts or on the root surface as rhizoplane fungi.

B1a2. In plant litter

Colonization of terrestrial litter in cold or temperate climates has been reported for a number of Ingoldian species, but water relations affecting *in situ* sporulation have not yet been studied. Even prior to Ingold's work in streams, Scourfield (1940) had detected conidia of *Tetracladium cf. setigerum* and *Volucrispora ornithomorpha* from leaf carpet washings in Britain. Bandoni (1972, see above), in Canada, isolated conidia of well over a dozen Ingoldian species from moist-incubated tree leaves in forest litter and from fallen *Brassica* leaves on a garden soil. Staurospores of other species were also observed. In 1981 he compiled over 30 names from such microhabitats. In the UK, Webster (1977) collected individual leaves at monthly intervals over one year from forest litter along a transect diverging at right angles from a permanent stream. Those from the furthest point (80 m) still yielded conidia of *Alatospora acuminata, Articulospora tetracladia, Gyoerffyella rotula, Tetracladium setigerum, Tricellula aurantiaca, Tricladium splendens, Varicosporium elodeae* and *Ypsilina* (as *Volucrispora*) *graminea,* but after laboratory submersion for four days at 10 °C. Also in the UK, Sanders & Webster (1978) reported the survival of 15 species of Ingoldian fungi on artificially or naturally colonized *Quercus* leaves in forest litter, on the basis of sporulation in distilled water, this time for one to seven days at 15 °C. Sporulation after moist incubation in both cases would have further substantiated the capacity for non-aquatic existence. In a much drier Mediterranean forest, of the *s. str.* morph *Alatospora acuminata* (with often one-armed conidia, as seen in interface sporulation) has been observed by Descals (unpubl.) in washings from forest litter. As in *Articulospora tetracladia*, there may be here more than one biotype.

Ingoldian fungi have also been found colonizing ephemeral accumulations of dead leaves and wood in tree holes (Gönczöl & Révay 1996). Sporulation here presumably takes place *in situ*.

B1a3. In soil

There are a few early records claiming the presence of Ingoldian fungi in soils which were supposedly away from streams. Hartig (1880) reported sporulation of *Mycocentrospora acerina* on laboratory-incubated soil, and Bessey (1939) obtained

conidia of *Varicosporium elodeae* from hemp seeds placed as baits on a soil suspension. These records need verification because of the possibility of contamination from the litter above. The latter fungus was also isolated from agricultural soils on which grew *Phaseolus vulgaris* (Parkinson & Thomas 1969). It should be interesting to do a thorough survey for sporulating mycelia of Ingoldian fungi in soils near streams.

B1b. In natural waters of various salinities

It is usually assumed that depressed sporulation in sea and brackish waters is due to salinity, but there could be other causes, such as the toxic effect of individual ions. The ability to sporulate in high levels of salinity among the few Ingoldian fungi so far studied depends on the species. Müller-Haeckel & Marvanová (1979b) showed that of eight tested in culture, only *Volucrispora graminea* sporulated in artificial seawater (49800 μS, 3.5 % salinity). Byrne & Jones (1975, our table 2) observed that sporulation of two species on Cornmeal Agar (CMA) cultures was severely affected by submersion for three to four days in seawater of various concentrations; but while one species stopped sporulating in 20% seawater mixtures with distilled water, the other still produced significant numbers of conidia in 30% seawater. In both species, total repression of sporulation at slightly higher seawater concentrations cannot be accounted for.

Table 2
Conidial production in cultures of two Ingoldian fungi submerged in increasing concentrations of sea water in distilled water (modif. from Byrne & Jones 1975)

	conidia/sq. cm.	
% seawater	*Heliscus lugdunensis*	*Tetracladium setigerum*
0	2500	1600
10	2400	800
20	700	0
30	600	0
40	0	0

Heliscella stellatacula is known from fresh water (E. Chauvet, pers. comm.), but it can colonize pine panels in estuaries (Kirk 1969). However, sporulation here was observed only after moist-incubation in the laboratory. There are still too few field records of this fungus to be certain about its typical habitat.

Nilsson (1958) saw conidia of *Heliscella stellata* and Willén (1958) of *Lemonniera aquatica* in brackish waters (the latter with 0.3% chlorides), but their source and viability were not determined. Müller-Haeckel & Marvanová (1979b)

later showed that when leaves taken from forest litter near a river were submerged for up to 14 days in a bay (where the electrical conductivity was only 3800-8300 (S/cm) eight species sporulated. It is not clear, however, whether sporulation was *in situ*. Jones & Oliver (1964, our Table 3) detected *in situ* sporulation by seven Ingoldian species on wood test blocks in tidal parts of a river. Again, the degree of response strongly depended on the species, as well as, in some cases, on the type of substrate. On the other hand, terrestrial hyphomycetes such as *Aspergillus niger* and *Stachybotrys atra,* as well as the marine *Asteromyces cruciatus* and *Dendryphiella salina*, sporulated even in pure sea water.

Table 3
***In situ* sporulation levels of seven Ingoldian fungi on wood baits, varying on a scale from nil (–) to high (+++) (modif. from Jones & Oliver 1964)**

	Pinus	*Fagus*
Alatospora acuminata	+++	+++
Anguillospora sp	–	+
Clavariopsis aquatica	+	+++
Lemonniera aquatica	–	+
Tetrachaetum elegans	–	+
Tetracladium marchalianum	+	–
Tricladium splendens	+++	+++

Varicosporina ramulosa, also a marine hyphomycete, produced atypical conidia if the seawater concentration dropped below 20% (Byrne 1978). We have no records on the viability of seaborne conidia in fresh waters. Their absence in terrestrial habitats needs an explanation.

B1c. Fresh waters

1. Lentic waters

These habitats have been much less intensively explored for Ingoldian fungi than running waters, probably in part because due to less turbulence there is less foam from where conidia can be isolated, and because substrata are less easily retrievable. Reinsch (1888), shortly followed by de Wildeman (1893), illustrated conidia of *Alatospora cf. acuminata, Clavariopsis aquatica, Lemonniera aquatica, Tetracladium* spp. (i.e. *T. marchalianum* and probably *T. setigerum*) and *Ypsilina graminea* from submerged substrata in lakes and ponds, on which sporulation had probably taken place. Suzuki & Nimura (1961) recognized eleven species on decaying leaves in harmonic lakes, but Ingoldian fungi were almost absent in dystrophic (humus-rich,

strongly acid) and acidotrophic lakes (rich in inorganic acids), and, surprisingly, no conidia were found in oligotrophic lakes. Sporulation, however, was induced only after laboratory submersion (i.e. in standing tapwater at 15-25 °C for at least 24 h).

2. Lotic waters

The first ever record of an Ingoldian fungus was probably , and not surprisingly, from a small stream: Brébisson, in 1836 (in de Wildeman 1895a), working with diatoms in Noron, France, illustrated what he believed could be «l'état primordial de quelque espèce du genre *Equisetum*», and which the latter identified as conidia of his *Tetracladium marchalianum* (but which could have been or included *T. setigerum*). Relatively small but permanent streams have since been the main source for the description of the *ca*. 270 species of Ingoldian fungi known at present. Sporulation in these waters need not be considered strictly underwater, due to aeration and turbulence.

Slow-flowing large rivers have only received attention recently. Chauvet (1997), through ergosterol analysis, stated that fungi (in our view most probably including non-Ingoldian species) can represent up to 96% of the microbial biomass in submerged *Platanus* and *Populus* leaves in the French Garonne. But species lists of Ingoldian fungi in large rivers are scarce. Organic matter colonization by Ingoldian fungi and their sporulation in sediment or in the hyporrheos, which are characteristic microhabitats of this type of rivers, have not been studied.

Most streams worldwide are temporary, but the activity of Ingoldian fungi here has received little attention. Taxonomists are probably not attracted by their low biodiversity, nor ecologists by their low abundance and hence low importance in decomposition processes. Margalef (1953) was the first to observe conidia of Ingoldian fungi in temporary streams (i.e. of the omnipresent *Tetracladium marchalianum* on the karst of the Mediterranean Balearic Is.). In a nine-month study of foam and submerged leaves and wood in a Moroccan seasonal stream, Chergui (1990) recorded 12 species, not many if compared with permanent streams, although factors other than seasonality of flow (e.g. high salinity, sparse riparian vegetation, etc.) may have negative influences. Variations in conidial loads in water of summer-dry streams have been studied with membrane filtration by Jaroscak & Suberkropp (1988); conidia were already detected one week after water started flowing, concentrations later reaching values of 1000 to 6000 per litre. More streams with temporary flow regimes need to be studied in detail before generalizations can be made.

Water in temporary streams may flow from several months to just a few days or even hours, as happens in streams subject to flash floods or in irrigation canals (Iqbal *et al*. 1995). This duration may be a major determinant of abundance and biodiversity of most Ingoldian fungi in such waters. Whether the colonization of newly incorporated substrata is primarily through conidial transport or by mycelial invasion in natural leaf packs and wood piles, or even prior to submersion, is not known.

As expected, there was little biodiversity of Ingoldian fungi was observed in the few studies done on temporary pools. Nilsson (1958) only recorded *Clavatospora longibrachiata* and *Lemonniera aquatica* in such waters in Sweden. Bärlocher *et al.* (1978) listed *Alatospora acuminata, Lemonniera* sp., *Tricellula aquatica* and *Tripospermum* sp. from *Acer* leaves and autochthonous herbaceous plants submerged as baits in vernal (snowmelt) pools in Canada; but aeroaquatic fungi, Mucorales and terrestrial hyphomycetes were more abundant.

3. Organically polluted streams

Nilsson (1964a) first remarked that Ingoldian fungi were absent in very eutrophic lakes and ponds. Cooke (1954 and later papers, see Jones 1974) recorded *ca.* 300 species of fungi from organically polluted waters, but Ingoldians were not isolated, and were thus classified among the lymaphobes. Other workers agree on this relative scarcity of Ingoldian conidia and species in even slightly polluted streams. For this reason, in our biodiversity studies we always sample from sites above the village furthest upstream. (Even here, domestic livestock may wallow around these waters and pollute them; but this effect has not yet been studied). The apparent sensitivity of Ingoldian fungi to untreated sewage waters remains in our view unexplained.

Suberkropp *et al.* (1988) analyzed total organic matter, phosphates, ammonium, total bacteria and coliforms above and below outflows from sewage treatment plants along the R. Erms in Germany. It may seem surprising that the seven species listed showed no clear response to this kind of pollution, with high conidial loads (18,000-20,000 conidia/l recorded on one occasion) present even in the polluted stretches. However, the authors believed that the lack of riparian vegetation in the clean sites upstreams as well as high water turbulence in the polluted sites obscured the effect of treated waste waters.

B2. Physiological conditions

Several environmental factors of a physiological rather than ecological nature, and which can thus be studied in the laboratory more easily, will now be briefly surveyed. Firstly it should be recalled that the effect of a change in any factor may occur after the treatment has concluded (induction, triggering or pre-conditioning effects) or while this takes place. For example, a colony may sporulate in water after a short dose of near UV radiation applied prior to submersion (Iqbal 1975) or, alternatively, sporulation may occur while near UV is applied to a severed submerged colony for several hours or days. (The diurnal periodicity and duration of the dose needed for sporulation are not known, but they may be species-dependent). Marvanová & Bärlocher (1988) successfully pre-conditioned *Taeniospora nasifera* for sporulation by drying the colonies (grown on oat flakes mixed with 2% water agar) prior to submersion.

B2a. Substrata

It is conceivable that saprotrophic Ingoldian fungi can grow and sporulate hydroponically, i.e. by only assimilating dissolved nutrients from the water column, solid substrata thus not being needed. In pure culture, we have observed vigorous growth and sporulation of *Tricladium splendens* for several days on the glass walls of aerated, continuous nutrient renewal chambers (see below). The next step could be to test for *in vitro* growth and sporulation with only stream water in the laboratory, or in dialysis bags in a stream. Nevertheless, the exercise may be ecologically irrelevant if we accept that mycelia in streams are always in direct contact with decaying solid organic substrata (in/on leaves or wood, or amongst periphyton covering mineral surfaces). Sladeĉková (1963) recorded at least 14 species of Ingoldian fungi in an experiment in which inert substrata such as plastics, porcelain and glass were submerged in streams. But they were only seen as conidia in scrapings off the periphyton which had developed on those. The mycelium could have assimilated nutrients through contact with the organic matter deposits. Curiously, there were significant differences in degrees of colonization between the different substrates used, which remain unexplained. For example, on porcelain, values were much higher (up to 20% of the periphyton community). But differences may have been caused by species interactions in the periphyton. Park (1974) only isolated one species (*Tricellula aquatica*) by dilution plating from fibreglass membranes previously used as baits in a stream and then macerated. We have no idea of the biodiversity of Ingoldian fungi in that stream at the time.

The presence and reproductive activity of Ingoldian fungi directly growing on rocky substrata (e.g. in epilithic communities) in streams have not been studied, although they do not seem to be present in large numbers.

Animal substrata should be abundant in streams, at least in certain seasons (e.g. at insect moulting). Conidia of *Mycocentrospora acerina* were once seen by Descals (unpubl.) growing on invertebrate exuviae. But evidence of colonization of macroinvertebrate remains by Ingoldian fungi is scarce. Conidia of *Gorgomyces hungaricus* were seen attached to the cuticle of live nematodes by Gönczöl & Révay (1985), though parasitism was not detected.

Allochthonous leaves are the most often recorded substrata, the first ever reference on broadleaved species probably being that of de Wildeman (1895b), who collected *Clavariopsis aquatica* on *Salix* leaves in a pond. There is evidence of mycelia colonizing some of these substrata preferentially, as reflected through differential sporulation levels in the laboratory. Ingold (1942) first observed that, of the species he originally studied, sporulation was generally more abundant on *Alnus* than on *Salix* leaves. Nilsson (1958) also noticed more sporulation on *Alnus* leaves than on those of *Acer, Salix, Quercus* or *Betula:* on other unspecified substrata sporulation was even less common. Cowling & Waid (1963) detected few Ingoldian species on *Eucalyptus* leaves, while *Casuarina* needles in the same streams bore several. According to Canhoto & Graça (1996), *Alatospora acuminata, Anguillospora longissima, Tetracladium marchalianum* and *Tricladium angulatum*

were rare on *Eucalyptus* leaves, but *Heliscus lugdunensis* was very abundant; the latter was, strangely enough, absent on *Alnus* and *Castanea*. Of seven species studied, Triska (1970) noted that *Campylospora chaetocladia* only sporulated on *Alnus* and *Salix* leaves. It is not known if the choice of leaves affects the levels of *in situ* sporulation for each species independently from colonization.

In highly acid moorland streams in England an apparently small but distinctive Ingoldian mycota sporulates on grasses (e.g. *Molinia*) and rushes (e.g. *Juncus effusus*) after laboratory incubation (Iqbal & Webster 1977). This often sporulates as irregularly distributed but very dense patches. Records of *in situ* sporulation should be interesting in these and other extreme habitats in view of Willoughby & Minshall's (1975) observations on the strong effect of very low water pH (three or less) on patterns of macro- and microconidial production (and therefore possibly on those of sexual reproduction) in *Variocladium* (as *Tricladium*) *giganteum.*

Ranzoni (1953) first detected Ingoldian fungi on wood and other lignified organs, and there are now numerous records on these substrata (Descals *et al*., in prep.). Archer & Willoughby (1969) even suggested that wood might be the only suitable substratum for some species. Whether *in situ* sporulation levels or patterns for any one fungal species are different from than on leaves, or even between wood species, is not known.

Endophytic colonization of live roots exposed to stream currents was demonstrated for nine species of Ingoldian fungi by Fisher *et al*. (1991). Pieces of bark and wood were submerged in aerated distilled water, and the fungi sporulated on them. The discussion under soil and litter fungi (as "geofungi", see above) applies here too.

The study of colonization and sporulation patterns on autochthonous vegetation, such as submerged and emergent macrophytes, aquatic mosses and lichens, etc., has been largely neglected. De Wildeman (1895a) first described *Tetracladium marchalianum* from submerged plants. Kegel (1906) saw interface sporulation of *Varicosporium elodeae* on decaying *Elodea* stems and leaves. Ingold (1959) recorded *Articulospora tetracladia, Polycladium equiseti* and *V. elodeae* on floating detached *Equisetum* internodes. But it was Kirby (1987) who proved underwater substrate colonization after serially washing or surface-sterilizing and then plating leaf particles of *Ranunculus penicillatus var. calcareus*. *Lemonniera aquatica* appeared in 75% of the isolates, with *Tetracladium marchalianum* and *Mycocentrospora acerina* also present. *In situ* sporulation was not recorded.

Seminatural agar media were first used as laboratory substrata by Kegel (1906) for inducing sporulation in *V. elodeae*. Karling (1935) later obtained sporulation of *Tetracladium setigerum* on CMA, PDA and Sabouraud dextrose agar. 2% MaltAgar (MA) was then used by Ingold (1942) and, with CMA, they have since been popular as growth media for the Ingoldian fungi. The former has the advantage that highly diagnostic colony and diffusible pigments are much more conspicuous than on weaker media; but sporulation is often poor when pieces are submerged in standing water.

As to effects of nutrient concentrations, it is well known that in many cases weak agar media promote sporulation in standing water. This was first observed by Tubaki (1958) in *Diplocladiella scalaroides,* which sporulated poorly on unsubmer-

ged 2% MA but heavily on 0.1% glucose-Czapek agar. Sporulation levels in 16 species of Ingoldian fungi were seemingly improved by Miura & Kudo (1970) when using a weak mineral medium (supplemented with 0.1% glucose and yeast extract). However the relatively low agar concentration (1.3 %) may have also increased the water activity of the medium and thus favoured sporulation; a control with the standard 2% agar could have been used. 0.1% MA is a common medium in some laboratories both for growth and sporulation in standing water. Suberkropp (1984, our Table 4) compared the sporulation of four Ingoldian fungi on leaf baits by three different techniques: *in situ* sporulation, submersion in stream water and contact with a mineral salt agar medium. The reason for using the latter technique was not explained, but in 19 out of 48 cases frequencies of occurrence were considerably higher than with the other two techniques; albeit with strong differences between species were observed.

Table 4
Suberkropp's (1984) mineral salt - agar medium (weights in g)

KNO3	2.50
K2HPO4	0.43
KH2PO4	0.34
MgSO4. 7H2O	0.49
NaCl	0.37
agar	15.00
dist. water	1 L

The possible effect of varying substratum nutrient levels of nutrients on hyphal density and consequently on conidiophore density and conidial production does not seem to have been contemplated.

Substratum nutrient concentrations seem to affect the incubation time needed to produce the first detectable conidium. Thus, Singh (1972) obtained conidia of *Pyramidospora constricta* after cultures had been grown for 20 days on 2% MA, but only for seven days on 0.1% MA. This aspect needs further analysis, as it is not known if sporulation starts sooner or if the sporulation rate is accelerated. Both mechanisms could operate. Maybe sporulation requires previous digestion of excess nutrients in the medium.

In aquatic sporulation, the effects of nutrient concentration and selection of nutrients in the substrata cannot be easily separated from those in the surrounding liquid medium because, whether leaves, wood or agar media are colonized, nutrients diffuse into the latter at unknown rates and proportions. Secondly, we do not know if developing conidiophores and conidia assimilate nutrients directly from the liquid medium or via the mycelium.

Nutrient concentration also affects reproductive morphology, as reported by Petersen (1962) for conidiophore branching complexity in slide cultures of *Tricellula inaequalis*. The composition of the medium and the nature of the effects were not specified.

In vitro, nutrient requirements may reflect the substratum preference or specificity for sporulation in nature. Thus the effect of choice of nutrients in agar media on sporulation levels should be investigated in more detail. For example, Miura & Kudo (1970) could not induce sporulation with their defined medium in two Ingoldian species tested, while eight failed on Yeast Glucose Agar (YGA) and MA. Nawawi (1974a) added 0.1% carboxymethyl cellulose to Miura & Kudo's medium and presumably improved sporulation in several species.

For unknown reasons, different plant substrata may sometimes enhance sporulation *in vitro*. Nawawi (1975) observed this in *Isthmotricladia gombakiensis* cultures grown on rice straw mixed with Oatmeal Agar (OMA) prior to partial submersion. No conidia were produced on submerged 2% MA. Bandoni & Marvanová (1989) succeeded in producing conidia in *Ingoldiella nutans* when *Lupinus perennis* stems embedded in water agar were colonized and later submerged in aerated water.

Agar concentration, as pointed out above, is another important factor to consider in sporulation. Although agar has 3% nitrogen and some of this may affect sporulation, its effect on the water activity of the medium is probably more important. Nilsson (1964b) made the unconfirmed statements that all freshwater hyphomycetes can produce conidia on water agar, although stauroconidia are then less branched and the arms diverge more or less in one plane, the latter effect probably being due to increased surface tension. Tubaki (1966) first observed that the *Tricladium* state of *Hymenoscyphus varicosporoides* was produced on water agar without further adding water. Several other cases are known. It should be noted however that below a certain concentration, and depending on the pH, agar will not set.

Jooste & van der Merwe (1990) found that *Flagellospora penicillioides* sporulated better in a leaf extract medium made up with 5% instead of 2% agar. This may seem surprising because the lower water activity at 5% might be expected to hamper sporulation, but perhaps the benefit was from the consequent drop in nutrient in the concentration of available nutrients.

Hyphal wounding

Invertebrate foraging and shredding and the mechanical action of water, as well as abrasion by suspended mineral particles in turbulent waters, must cause substantial wounding of fungal thalli, particularly of exposed reproductive structures. This might be an important environmental factor in sporulation dynamics, and deserves some attention.

In pure culture, there is ample evidence among hyphomycetes which sporulate aerially that cutting holes in colonies can induce conidial production on the newly exposed surfaces. It has been postulated that hormonal substances are responsible for this and that they accumulate at the cut hyphal ends (see Nilsson 1964a). Among Ingoldian fungi, hyphal wounding is involved in sporulation techniques based on cutting out portions of agar cultures or colonized leaf discs for submersion. Aquatic sporulation on submerged cut surfaces often occurs in a rather unpredic-

table way, conidiophores appearing as dense forests, clustered or scattered depending on the species.

The effect of hyphal wounding in Ingoldian fungi needs to be studied experimentally and separated from that of mere contact with free water. In techniques whereby growth and sporulation take place on undisturbed substrata in the same culture chamber (e.g. some of the water and nutrient renewal techniques discussed below), there is no wounding, and this may affect conidial production levels.

B2b. The liquid medium

B2b1. Dissolved nutrients

In streams, it is claimed that up to 30% of the leaf dry weight may be lost by diffusion within 24 h of submersion (Moss 1980). Although this may be exagerated (E. Chauvet, pers. comm.) we do not know what part of the leaf bioncass is available to fungi as nutrients, nor whether other factors operating at low concentrations enhance (or even induce) fungal growth or sporulation. Kaushik & Hynes (1971) showed that dissolved nitrogen could be incorporated into proteins by fungi growing on decaying leaves; and numerous subsequent papers have dealt with nutrient dynamics in decomposition.

In the laboratory, Hartig (1880) first obtained sporulation of an agar culture of *Mycocentrospora acerina* in fruit juices on a microscope slide. Tubaki (1957) observed sporulation in *Articulospora tetracladia* grown in a rich medium (2% malt solution). This is unexpected, as in high nutrient concentrations Ingoldian fungi will normally grow but not sporulate (Nilsson 1964a). Greathead (1961) furthermore noted that agar cultures of various species sporulated better in stream than in distilled water; and Shearer & Hewings (1979) first used a dilute salt solution for inducing sporulation of 17 species on field-colonized leaves. A certain ionic concentration and balance seem to be necessary for optimum sporulation, but the requirements for individual species are not known.

As substrata exposed for long periods in streams (e.g. leaf and possibly to a lesser degree wood baits) lose part of their nutrients through leaching, stream water or weak nutrient solutions should in principle be more effective than distilled water for inducing *in vitro* sporulation. Ciferri (1959) detected some sporulation enhancement when skeletonized leaves were incubated in 1:100 and 1:1000 dilutions of Berthelot's mineral solution. Suberkropp (1984) first used filter-sterilized stream water for his incubations, but later (Suberkropp, 1991, our Table 5) maintained sporulation of *Anguillospora filiformis* and *Lunulospora curvula* on *Liriodendron tulipifera* leaf discs for almost a month in an aerated dilute mineral solution. This was replaced every other day (see below). Such artificial mineral liquid media have been introduced into sporulation protocols in recent years, but the extend to which their ionic composition approaches that in the streams from which the fungi are derived is not discussed.

Table 5
Suberkropp's (1991) mineral salt sporulating medium (weights in g)

KNO3	0.1
K2HPO4	0.2
MgSO4	0.1
CaCl2.2H2O	0.1
dist. water	1 L
pH 7 (buffered)	

There seem to be unknown factors which play a role in sporulation at very low concentrations. Nawawi (1973, 1974b), for example, obtained good sporulation in *Flabellocladia tetracladia* and *Dendrosporomyces splendens* when sterilized *Hevea* petioles were added to the water in which sporulation was induced; macronutrients in the petioles would clearly be too dilute to elicit such a substantial response.

B2b2. Water activity

Some aspects of the water activity of gelified substrata have been mentioned above. The balance between water activities in the substratum and in the surrounding liquid medium is probably one of the most important factors in sporulation, but it seems not to have been studied.

B2b3. Carbon dioxide concentration

In streams, Woelkerling & Baxter (1968) observed the highest numbers of species and conidia in streams with CO_2 levels below 50 ppm. In the laboratory, Ciferri (1959) had previously observed that when skeletonized leaves naturally colonized with unspecified Ingoldian fungi were submerged in renewed water and subjected to an external supply of CO_2, sporulation ceased, but resumed if CO_2 was replaced with air or oxygen.

The accumulation of metabolic rather than external CO_2 does not however appear to harm sporulation. Webster & Towfik (1972) submerged agar colony discs with *Articulospora tetracladia* or *Lemonniera aquatica* in tapwater in a bottle and recirculated the atmosphere in a closed system for three days by means of a pump. The CO_2 concentration was double that in open control bottles, but no significant differences were observed in sporulation. In a second set of experiments with the same fungi, four bottles containing agar discs with either of these fungi were connected in series and air was bubbled from one end for three days. It was thought that the concentration of CO_2 would gradually increase down the line and sporulation rates, if affected, would drop proportionately. But these were not sig-

nificantly different. However, *A. tetracladia* may not be the best choice for future work on the effects of aeration and CO_2, as it has since been found to be more common in stagnant waters than other Ingoldian fungi, and this could imply an atypical tolerance to anaerobiosis or noxious gases).

B2b4. Forcible aeration, agitation and turbulence

Ranzoni (1950) first observed that when *Anguillospora longissima* was cultured in weak nutrient media (with 1 g/L glucose) mechanically shaken at 28 °C, 173 mg dry weight of biomass were produced as opposed to 38 mg in standing water and 112 mg in aerated water (but at 24 °C). The fungus was incapable of sporulating at 28 °C, which was not surprising if the fungus was correctly identified; but sporulation at 24 °C was more likely. In this case, and from what has been mentioned above, the effect of either aeration or agitation on vegetative growth would not be clearly distinguished from that on conidial production.

Ingold (1958) seems to have been the first to apply forcible aeration to enhance sporulation in the Ingoldian fungi when he described *Lemonniera terrestris, Anguillospora crassa* and *Dimorphospora foliicola*. This technique has since become standard for conidial production in many species of Ingoldian fungi.

It should be noted that in quantitative work the rates of entry of air into the sporulation flasks are usually expressed as mL/min., but, as the liquid volumes and surface areas vary between authors and even experiments, such a unit of measurement is meaningless unless correlated with the actual effect on either aeration rate or degree of turbulence or just of mixing of the medium (parameters which are relevant but probably not easy to measure).

The response of fungal vegetative growth in leaves to increased aeration is unknown. Nor do we know the response of decomposition rates, which are probably afeccted by vegetative growth and enzyme release. Sporulation on field-colonized leaves and wood also seen to respond to forcible aeration. For instance, Bärlocher (1982) recorded 41 species on *Quercus, Larix* and *Picea* leaves; and Shearer & Webster (1991) placed field-colonized twigs in 180 × 18 mm glass tubes and bubbled air from the bottom for two days, single twigs yielding up to 700,000 conidia belonging to eight species.

Attempts to explain the effects of forcible aeration on conidial production were not initiated until 14 years after Ingold's first report (Webster & Towfik 1972; Webster 1975). For the first paper, the authors submerged discs with 2% MA cultures of *Articulospora tetracladia, Heliscus lugdunensis, Lemonniera aquatica, Tetrachaetum elegans* and *Varicosporium elodeae* in aerated tap water. (In their experimen, the water was changed daily, which may have significantly enhanced and prolonged vegetative growth and conidial production). When the rate of aeration was raised from 100 to 1000 mL/min, the sporulation rates doubled. Controls in standing water were not used, probably because of problems with conidial harvesting due to adherence to the walls.

In a separate experiment, and to eliminate the effect of nutrient supply or diffusion from agar media, mycelial pellets produced in 2% malt solution in flasks on a shaker were washed and then induced to sporulate in tapwater (again changed daily), with aeration rates at 100, 500 and 1000 mL/min. Significantly more conidia were produced at the highest rates. The quantity of nutrients stored in the parent hyphae may vary depending on the concentration of the solution in which the pellet is produced. With 2% malt solution was used, sporulation may have lasted longer than with poorer media.

Ingoldian fungi can be capable of sporulation with very little dissolved air in the water (Webster & Towfik 1972 fig. 3). Agar cultures of *Articulospora tetracladia* and *Lemonniera aquatica* were exposed to air-nitrogen mixtures. Sporulation in almost pure nitrogen was still 75% that in air, and it was concluded that sporulation was unlikely to have been increased exclusively by a rise in air (and presumably oxygen) concentrations. In a second experiment, the air solution rate in the bottles was greatly increased by reducing the size of the air bubbles with a sintered glass bulb; but with large bubbles (coming out of a hypodermic needle) there was unexpectedly more sporulation. As the larger bubbles caused more turbulence, sporulation enhancement was attributed to a mechanical effect.

Because turbulence could be provided by just agitation, Webster & Towfik (1972, Tables 5-8) compared the effect of agitation and forcible aeration on the sporulation of 2% MA cultures of *Varicosporium elodeae, Heliscus lugdunensis, Lemonniera aquatica* and *Articulospora tetracladia* in tapwater. As the aeration rate was increased at a constant stirring rate, sporulation also increased, but the agitation effect was proportionately smaller, probably because aeration also caused agitation. As the stirring rate was increased at a constant aeration rate, sporulation again increased, presumably due to a rise in turbulence. Studies comparing the effects of aeration and agitation in separate containers have not yet been performed.

In pure culture, as forced aeration has a greater risk of contamination and requires pumping and the sterilization of the air, it would seem much more convenient instead to use agitation, but the technique has not become popular. It has been said that conidial yields in stirred flasks is not as high as in aerated ones (Bärlocher, pers. comm.), but there are no experimental data to support this.

On natural substrata fungal sporulation appears to respond to agitation, but the two cases we refer to below did not test for this. Khan (1981), obtained sporulation on field-colonized leaf disks placed in sterile stream water in Petri dishes on a rotary shaker at 30 rpm for two days. Baldy *et al.* (1995) studied community structure and sporulation rates with leaves incubated in flasks with filtered river water placed on rotary shakers.

Aeration and agitation have also been applied at the mesocosm level in leaf decomposition experiments. Thompson & Bärlocher (1989) studied the effect of pH on leaf decomposition by placing colonized leaves in a circulating channel filled with unchanged water flowing at 45 cm/s. The pH was adjusted every other day with ammonium sulphate and sodium hydroxide (both of which may have independently affected sporulation levels). Hamilton (1973) combined agitation with aeration when he incubated field-colonized leaves in an aerated tank ($40 \times 28 \times 15$

cm) equipped with a recirculating pump. However, in neither case were levels nor duration of sporulation determined.

We are unaware of any explanation for the enhancement in sporulation of Ingoldian fungi through turbulence, but it is worth recalling that during growth and maybe even sporulation, gaseous or dissolved staling compounds (e.g. organic acids, which can drastically change the pH of the liquid medium) are exuded from fungal walls. In standing water they would concentrate around the hyphae and so could interfere with sporulation. If the water is mixed (even without renewal), which could be achieved through turbulence, staling compounds around the fungal walls would become diluted, and sporulation could then proceed. Other factors could also intervene, as sporulation is found to linearly with turbulence when only a little agitation would be expected to reduce the concentration of staling compounds around hyphal walls.

It should be interesting to check if a third source of enhanced sporulation under turbulence could be the increased mycelial biomass produced.

Webster (1975) observed two mechanisms which resulting in enhanced sporulation due to increased aeration. In five species he studied there was a significant rise in the number of conidiophores per unit surface of mycelium; and in six species, there was a shortening in the duration of individual conidial development by several hours. In *Lunulospora curvula*, however, the timing was not altered. He also found that forced aeration decreased the number of conidiogenous cells per conidiophore. In *Lemonniera aquatica* there were fewer phialides per conidiophore at higher aeration rates than at lower ones.

Under non-axenic conditions, Greathead (1961) observed an extension the total duration of sporulation of various species on decaying leaves from two days in standing water to seven days if it was aerated. The time taken by the mycelia to initiate sporulation and the total conidial production were not given. In a culture of *Casaresia sphagnorum*, Webster *et al.* (1993) also observed that the first conidia were formed after ten days in standing water, but only after three days if this was aerated. Presumably it was the duration of conidial development which was shortened and not the previous induction period.

Webster (1975) reported that in *Varicosporium elodeae* the mean number of branches per conidium dropped from 7.2 to 1.8 as aeration was increased from 0 to 1000 mL/min. He claimed that branches did not break off due to turbulence and the conidia were thus assumed to secede earlier and to be consequently less branched. However, in conidia of this species where branches are held at delicate insertion points, it seems more probable that turbulence increases the degree of conidial fragmentation. Indeed, conspicuous scars are frequently seen in conidial branches of *V. elodeae* (cfr. Webster's 1975 fig. 1). It is also possible that turbulence stimulates the production of secondary conidia, and these are normally smaller and less branched. In species where all the conidial elements are similar, it may be difficult to distinguish between primary conidia, part-conidia resulting from fragmentation and secondary conidia (see under Reproductive Properties above). Species with more robust conidia should be used for this kind of studies.

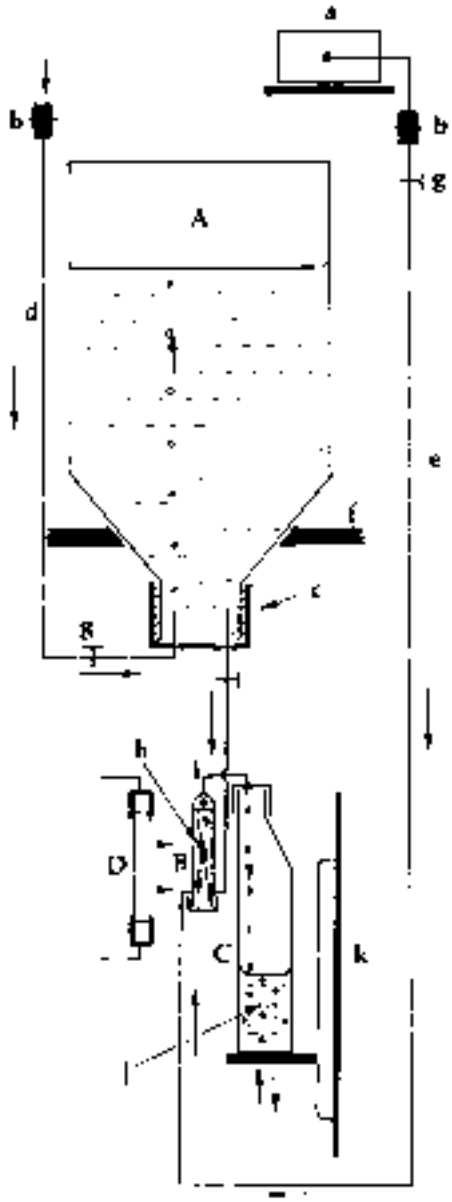

Figure 1.—Culture chamber with continuous medium renewal. A: carboy with nutrient solution. B: culture chamber. C: Collection bottle. D: NUV and daylight lamps. a: air pump; b: air filters; c: Multi-fit screw top; d: constant head tube; e: aeration tube; f: support for carboy; g: flow valves; h: glass rod with mycelium; i: U-tube for effluent; j: conidial suspension in fixative; k: slide rack for hydrostatic regulation of nutrient flow.

Morphological and ontogenic effects due to aeration which do not necessarily affect conidial numbers have also been studied. Webster (1975) found that the conidiophore length was reduced in a highly aerated culture of *Lemonniera aquatica*. Marvanová (1988) also observed an inverse effect of aeration on conidial length: in *Colispora elongata* this was 42-93 (m without aeration *vs*. 35-55 (m in aerated water. She interpreted the shortening as premature secession. The same phenomenon was seen in *Colispora curvata* by Nawawi & Kuthubutheen (1989).

Marvanová (1972) believed that in *Calcarispora hiemalis,* detached conidia could be more readily dislodged in the turbulent waters of a stream than in standing water. However, the ease of dislodgement *per se* probably does not affect conidial production rates. It is however not known if early dislodgement affects the mode of conidiogenous cell proliferation, i.e. whether poor dislodgement in normally percurrent cases induces sympodial regrowth.

B2b5. Water renewal

The term «water flow» is often used when water is being replaced. But in the presence of turbulence, any point on the mycelial surface is subject to flow without the water being necessarily renewed. To avoid confusion, we use the term «water renewal» for cases when the water is replaced.

In the above experiments with *Articulospora tetracladia* and *Lemonniera aquatica* Webster & Towfik (1972) observed that when agar colony discs were aerated in tapwater for seven days and this was changed daily, sporulation peaked at three to four days but then dropped drastically. This was not explained, but nutrient exhaustion may have been the cause. (i.e.: water may not have contained enough nutrients). On leaf or wood substrata, it is believed that such an effect would not be so drastic.

Descals *et al*. (1976) subsequently observed that pieces of culture on 2% MA of *Actinosporella megalospora* and *Porocladium aquaticum* sporulated very sparsely or not at all in standing or aerated distilled water. Conidial malformations were frequent, and it was suspected that staling factors (possibly including excess nutrients) were not being eliminated from the mycelium or from the water. A slide chamber was thus designed to provide for continuous water renewal and at the same time to enable the monitoring of its microscopic effects on conidial production. Two tropical species, *Brachiosphaera tropicalis* and *Clavariana aquatica*, were included in the study for reasons exposed in the above reference. It was believed that by observing conidiogenesis clearer species differences could be detected. Sterile distilled water was fed by gravity flow at the arbitrarily chosen rate of *ca*. 1 l/day. Sporulation in all four cases was good, even in *Brachiosphaera*, which had been seen to produce conidia in mucilage drops in air. In all cases sporulation eventually stopped, presumably also due to nutrient diffusion from the agar and eventual exhaustion in the hyphae. (Nutrient media were unfortunately not supplied later to test this). There were still some malformations (i.e.: mainly an abnormal elongation of the conidial central bodies in *Actinosporella* and *Porocla-*

dium), probably caused by either lack of aeration or by the insufficient drainage of unknown factors. No such effect has ever been observed in conidia of *A. megalospora* in nature, nor in the two tropical fungi sporulating within the chamber.

The type of chamber described above is somewhat cumbersome to set up for routine sporulation studies (the coverslips have to be sealed with hot paraffin, care has to be taken to avoid bacterial contamination), the eddy currents in the chamber may not result in sufficient mixing due to poor design, gas exchange is by diffusion and thus probably inadequate, and microscopic resolution is not satisfactory due to the excessive depth of the chamber (*ca*. 2 mm). An improved design in which this depth is not a limiting factor will be tested.

Commercially produced water renewal chambers similar to that described above were later used successfully by Nawawi (e.g. 1976b) for describing other tropical fungi.

Sanders & Webster (1980) designed a similar chamber for studying the sporulation response of 17 species of Ingoldian fungi to renewal rates in distilled water at 0, 5, 10, 50, 100 and 200 mL/h (the latter two calculated to approach those of fast flowing streams). Unfortunately, seven species scarcely sporulated in standing water, although all of them were known to do so when colony discs were submerged in larger volumes of water in Petri dishes. All sporulated well at 5 mL/h. In six of those species, sporulation rates did not increase at the higher renewal rates, but in *Tricladium splendens* where sporulation was significantly improved (by 25%) at 10 mL/h. *Heliscus lugdunensis* yielded large numbers of conidia even in standing water. Their shapes were not given, but if they lacked the distal knobs, they may have been produced aerially even before submersion. *Articulospora tetracladia* (*sensu* Ingold 1942) increased conidial production from just traces at 5 mL/h to maximum levels at 10 mL/h. But *Tetracladium setigerum* did not sporulate until the rate reached 50 mL/h, which was surprising because in nature it sporulates on unsubmerged forest litter). In seven other species sporulation gradually increased with the rate of water renewal, with maxima at anywhere from 10 to 100 mL/h.

Further experiments including other species should be made, but an interesting conclusion can be drawn. While in the original design by Descals *et al*. (1976) there was some turbulence in Sanders & Webster's chamber conditions approached laminar flow. The response to renewal was however very good in all species except *H. lugduneusis*. It seems therefore seems that turbulence, at least in this situation, is not needed. One could in the future possibly distinguish the effect of the elimination of staling metabolites from that of removal of excess nutrients from agar and hyphae by maintaining a constant nutrient concentration in the chamber but without a liquid outflow.

Leightley & Eaton (1977) followed soft rot development in wood caused by the marine ascomycete *Halosphaeria mediosetigera* in seawater continuously renewed under axenic conditions in a «decay chamber» (Wild Heerbrugg) with a greatly reduced inner depth (20 (m). Sporulation parameters were not studied, but a similar chamber could be adapted for such purposes.

There are no laboratory records on the effect of water renewal on sporulation

under non-axenic conditions. If the chamber used by Descals *et al*. (1976) became contaminated, bacteria were seen to proliferate on the surface of conidial cells showing lysis. Lack of air in the chamber may have created favourable conditions for bacterial proliferation. If this were actually the case, aeration should be introduced into future designs. In well aerated streams, however, sporing structures of Ingoldian fungi appearing on leaves do not seem to be colonized by bacteria.

B2b6. Hydrostatic pressure

There is apparently no information on the effect of depth of the water column on sporulation in the Ingoldian fungi, a factor which should be important in large rivers and especially in lakes and reservoirs. Willoughby & Archer (1973) proved colonization of twig baits at 1.5 and 6 m depth in a lake, but did not check for *in situ* sporulation. If sporulation is repressed by increased depth, freshly fallen substrata would have to be colonized by mycelial invasion from neighbouring substrata, or else by a constant supply of inoculum settling from above. Alternatively substrata may be previously colonized in forest litter, or while floating on water. Inoculation by aquatic invertebrates (which may carry conidia or hyphae on their surfaces or on mouthparts or faeces) is also conceivable.

If hydrostatic pressure proves to be deleterious for sporulation, we should probably ask ourselves if submersion techniques in the laboratory are necessary or even recommendable in some cases. There is now evidence for many species of normal interface sporulation under semisubmerged conditions in Petri dishes, and their performance could probably be greatly improved by using open-air perfusion systems such as those described below. In shallow streams at least, most sporulation probably occurs principally at the numerous interfaces created by turbulence rather than underwater.

Two features of field sporulation need explanation:

1. Suppressed sporulation

Ingold (1942) first observed that of the 16 species he studied, *in situ* sporulation on leaves was repressed in the colder months. However, when the same leaves were placed in dishes with water, large numbers of conidia were formed after a few hours. This drastic enhancement in sporulation response when colonized leaves are taken out of the stream has been corroborated by a number of authors (and quantified by Suberkropp 1984). (We assume that a similar response would be elicited by fungi colonizing wood but there are no comparative data). The rise in temperature need not be the only cause as, according to later publications, enhanced sporulation also happens if laboratory incubations are performed at low stream temperatures. However, higher temperatures during transport to the laboratory could conceivably trigger sporulation in some unknown way.

Willoughby (1978) suggested that increased conidial production might be due to nutrient enrichment or possibly to the breaking of some kind of natural stasis, as

happens in soil sporostasis. Assuming that either the nutrients or the staling compounds involved are water soluble, these hypotheses could be tested in the laboratory by setting up a continuous renewal system with two containers connected to each other; the first one could have either nutrient solutions similar to those found in streams, or else the presumed source of the staling compounds (e. g. pure cultures of resident fungi or bacteria, or decaying substrata). The effluents would then flow into the second container with the fungal culture under study, and the effects on sporulation levels could be determined through conidial counts in the outflow. If sporulation enhancement is foun to be due to enrichment and we are only interested in recording biodiversity, we should search for optimal nutrient concentrations in our incubations; bacterial proliferation could then be controlled with antibiotics. If it is due to a breaking of sporostasis, then liquid and/or gas renewal would have to be incorporated into the sporulation vessel.

It is also possible that invertebrate feeding on conidiophores and conidia checks their numbers in streams. Neither this phenomenon nor that of the seasonal effect on levels of field sporulation (i.e. the lack of suppression observed by Ingold in the summer months) have yet been properly approached.

2. Increase in total conidial loads after rainfall

Ingold (1965) observed that spore loads in streams increased drastically after rains, when water discharge rose. This is now a well established fact. An analysis of the composition of the spora in water (as seen on membrane filters) is however needed. Foam cakes accumulating during spates contain an incalculable number of tiny spores of unknown nature and source. Some at least are conidia of *Acremonium* spp. They become attached to the Ingoldian conidia and, when plated out, quickly produce mycelia which sporulate on the isolation plates. Some species —diagnostic stauro— and scolecoconidia in foam belong to fungi typically colonizing terrestrial habitats. Others are of Ingoldian fungi, but they could come from outside the water. (If they were formed prior to flooding, their viability may have been affected). Other Ingoldian conidia will of course be from already submerged substrates. Once any substratum, whether submerged or not, is exposed to fast-flowing, turbulent water, mycelia of Ingoldian and most probably other fungi too, will produce a first crop of conidia within as little as four hours. But it is not known to what degree the sporulation of individual species responds to environmental factors in streams, i.e.: what proportions of different species would be found prior to and after rains. If mycelia in pure culture quickly increase their biomass as a response to aeration, they presumably do likewise in streams, and would soon add further crops of conidia to the water. A spate may thus have a delayed effect on conidial loads.

SPORULATION TECHNIQUES

In standard identification procedures, the Ingoldian fungi are first grown on nutrient agar media, which normally have low water activities. Colonies here develop

cultural artifacts which may be useful for descriptive purposes but which are not obvious in nature, e.g. aerial mycelium, pigment accumulation, zonations, production of survival structures associated with excess nutrients, etc. Sporulation is mostly absent, and to induce this, a portion of the colony is transferred to a liquid medium. Phenomena such as massive conidial production often occur, which may facilitate sampling for diagnostic work but which are likewise not seen in continuously flowing waters. There is also in many cases a dissolution of accumulated secretions from the mycelia, which, as mentioned above, are known to change for example the acidity of the liquid medium. It is assumed that they do not affect ontogenic and morphological features. Such cultural conditions probably approach those in forest litter, living plants or soils, where resident fungi tolerate (or may even be adapted to) alternate wetting and drying, but certainly not those in streams, where mycelia may spend most of their existence under water. When inducing sporulation Ingoldian fungi in pure culture, some mycologists have attempted to introduce more stream-like conditions in their sporulation techniques. These are surveyed here, including some of our more recent work, still in progress.

Techniques are based on providing moist air (for aerial sporulation), microlayers (for interface sporulation) or submersion of mycelia (for underwater sporulation). An isolate of a presumed Ingoldian fungus may sporulate in one, two or all three microenvironments. These should therefore be present in an ideal sporulation chamber; but as this does not yet exist, other environments should be made available to the fungus if standard semisubmersion in Petri dishes with distilled water fails. Some basic techniques and protocols have been detailed in Descals (1997) in an introductory laboratory manual.

I. Sporulation on unsubmerged mycelia

These techniques promote aerial or interface sporulation.

Colonies of most Ingoldian fungi grow slowly and are therefore kept in Petri dishes sealed with adhesive tape to reduce evaporation and contaminations. However, under these conditions, although the media may be of low water activity, the degree of air moisture saturation may be enough to induce water droplet formation, especially if there are marked changes in temperature during incubation. We cannot them say if conidia are formed aerially, at an interface or in condensation drops.

A few species, such as *Lemonniera terrestris*, *Mycocentrospora acerina* (various records), *Calcarispora hiemalis* (Marvanová 1972) and the basidiomycete *Fibulotaeniella canadensis* (Marvanová & Bärlocher 1988), sporulate freely on unsubmerged substrata, (although some also sporulate in standing or aerated water). Conidia in the latter species, as well as in others, are produced in mucilage drops. The function of mucilage in this connection is unknown.

For aerial sporulation, high moisture levels are probably always needed. Neergaard & Newhall (1951) used sulphuric acid at various concentrations to create environments with different levels of relative humidity. Sporulation on agar cultures

of *Mycocentrospora acerina* increased as the relative humidity rose from 80 to 97%. Such studies should be extended to other Ingoldian fungi and may thus help define them with a sound physiological criterion.

On unsubmerged nutrient agar media it is however generally accepted that most Ingoldian fungi do not sporulate or do so sparsely or atypically or just differently, usually with a reduction in number and/or size, or even abscence of conidial branches (e.g. *Heliscus lugdunensis*, Ingold 1942). Petersen (1961) also observed that conidia of various species, when produced out of water, were more variable in shape. The effects of aerial sporulation on the morphology of conidia or other reproductive parts of Ingoldian fungi when grown on natural substrates has not been studied extensively.

Newhall (1944) encouraged sporulation (probably aerial or interfacial) in cultures of *Mycocentrospora acerina* by submitting them to alternating phases of high relative humidity and dessication. Similar results for this fungus were obtained on colonized leaves by Viennot-Bourgin (1955). If the water was not replaced, the effect cannot be explained.

Westerdijk & van Luijk (1924, in Newhall 1944) obtained conidia of this same species by inverting an agar slab bearing the colony in the same Petri dish. Conidia appeared on the freshly exposed surface, but the reason for this is also unknown.

II. Sporulation in contact with liquids

The remaining techniques involve exposure to either free water or nutrient solutions. When placing a colonized substratum in contact with liquids, the ratio of mycelial mass to volume of liquid (or, for short, the mass/volume ratio) seems to have a strong effect on sporulation levels, but the reason for this has not yet been proven or explained. Low ratios are especially recommended in non-axenic conditions, where bacterial contaminants and their diffusible toxins accumulate quickly. Relatively high ratios are unavoidable in water microlayers, but also if mycelia are fully submerged, e.g. in hanging drops. They have sometimes been used in aeration and agitation techniques (e.g. 25-30 agar colony discs in 100 mL water; see Webster & Towfik 1972).

A) Contact with water

A1. Unchanged water

A1a. Standing water

A1a1. High mass/volume ratios

Srivastava (1958) considerably improved sporulation in *Mycocentrospora acerina* by laying a plastic sheet on an open culture in a Petri dish and then exposing it to NUV for two days. If the plastic sheet had any effect on sporulation this was probably because water condensed underneath and created a sort of microlayer.

Nawawi (1976a) obtained conidia of *Laridospora appendiculata* by simply covering the untouched colony in the Petri dish with a thin film of water. There was no mention of having drained this.

Van Tieghem cells have been used to create submerged conditions and to enable the microscopic observation of conidiogenesis and other processes. Kegel (1906) just used such cells for observing conidial germination in *Varicosporium elodeae*, but it was Ingold (1942) who first applied hanging drop techniques to describe sporulation in his aquatic hyphomycetes, especifically in *Flagellospora curvula*. Ranzoni (1953), Greathead (1961) and a few other workers have studied conidiogenesis in several species with this technique. Conidial malformations were reported by Greathead (1961), who thought that they were due to the accumulation of gaseous staling compounds (e.g. CO_2), as the opening of lateral vents was claimed to have solved the problem. Soluble metabolites or nutrients probably also accumulate and interfere with sporulation. Hanging drops have two further disadvantages: easy contamination and poor resolution except immediately below the coverslip.

A1a2. Low mass/volume ratios

Incubation in larger volumes of standing water to encourage sporulation in the higher fungi has probably been used since the earliest workers first discovered aquatic sporulation. Ingold (1942) used this technique on skeletonized *Alnus* or *Salix* leaves and on strips of agar media. Single leaves may produce up to 300,000 conidia in a single submersion exercise (Nilsson 1964a). The technique enables direct observation with the dissecting microscope, and possibly even with the compound microscope if objective lenses with a long working distance are used. (These should preferably not invert the image in order to facilitate conidial manipulation, for example when isolating spores. Dissecting microscopes are now available which have a supplementary objective with long working distance mounted on the same turret). The substratum is placed in the dish and water added up to the surface. Conidia can then form aerially (or possibly in condensation drops), at the interface or underwater. Submerged sporulation in some species sometimes only occurs on tiny pieces of mycelium accidentaly separated from the colony when this is submerged. This effect is probably caused by a more thorough dilution of staling compounds which had pressumably accumulated in the colony.

When incubating in standing water, passive gas exchange is not impeded and convection currents may slightly mix the water, but there is no turbulence, no nutrient replacement nor any elimination of excess soluble nutrients or staling compounds. Neither does this technique allow for representative sampling of detached conidia due to their stickiness, unless for example cellophane is first placed under the substratum: conidia would settle and if they adhere to the cellophane, counts could then be added to those of floating or suspended conidia. Alternatively one could may be avoid adherence by coating the inside of the dish with silicone or a grease, as suggested above for aeration flasks.

A1b. Turbulent water

Techniques involving turbulence have been discussed under Environmental Conditions above. Conidial stickiness can be an unsolvable handicap here, as conidia may remain in suspension for many hours, or even days. The air space under the lid should be reduced to a minimum to avoid conidial loss due to any bubbles bursting. As with standing water techniques, the effect of the mass/volume ratio on the quantity of conidial production has not been studied.

A2. Water renewal

A2a. Periodic renewal

A2a1. Non-aerated water

Sridhar & Kaveriappa (1984) submerged field-colonized leaf pieces for 60 days in Petri dishes, renewing the water every other day. Twelve species of Ingoldian fungi were detected. In pure cultures, Bandoni & Tubaki (1985) greatly improved sporulation in *Cladoconidium articulatum* on Malt Yeast Peptone Agar (MYP) by flooding the colonies in Petri dishes and decanting the excess water. *Naiadella fluitans* responded similarly when agar cultures were flooded with distilled water and drained several times (Marvanová & Bandoni 1987). *Arborispora paupera* on 2% MA pieces wetted intermittently also sporulated heavily (Marvanová & Bärlocher 1989). Presumably the water had been decanted. In a study on biodiversity, Dubey *et al*. (1994) recorded 45 species on field-colonized leaves submerged in water in Petri dishes, renewing the water every day for two to six weeks. Gönczöl & Révay (1996) reported that sporulation in *Colispora cavincola* was nil on 2% MA, and late and sparse if colony strips were aerated in water for three to four weeks, but greatly improved if strips were partially submerged or if the colony was simply soaked in sterile distilled water for a few days and then moist-incubated, presumably after decanting. This apparent enhancement through soaking and draining could imply the removal of staling metabolites or of excess nutrients. No data were provided in the above contributions on the variation of sporulation rates over time.

It would be interesting to see if by simply using washing techniques we could induce aerial or interface sporulation in fungi believed to sporulate only under water could be induced.

A2a2. Forcibly aerated water

Renewal with forcibly aerated water has been discussed in some detail above with regard to Webster & Towfik's (1972) experiments.

A2b. Continuous renewal

A2b1. Without forcible aeration

Under non-axenic conditions, Marvanová (1968) obtained good sporulation of *Lemonniera centrosphaera* by means of an open-air perfusion technique: water was dripped from a tap onto an MA culture for three to four days in an open dish.

The observation chamber of Descals *et al.* (1976), discussed above under Environmental Factors, was based on continuous water renewal, but this time under submerged conditions.

Marvanová & Bandoni (1987) later used a closed perfusion chamber (adapted from Marvan *et al.* 1979) where distilled water was very slowly irrigated (at 5 ml / 8 h) to induce sporulation of an agar colony of *Naiadella fluitans*. Sporulation was abundant (although it also occurred in standing water). Axenic conditions were presumably kept. The technique enabled microscopic observation, but there is no mention of conidia being harvested for counts. There is no explanation for the extremely low rate of water renewal used. If this technique proves to be effective, it may simplify the design of future sporulation systems, as much smaller reservoirs would be needed.

A2b2. With forcible aeration

Bengtsson (1992) improvised a «stream microcosm» for observing bacterial and fungal growth, conidial production and fungal/bacterial interactions in dual culture on leaves. Sealed staining jars ($58 \times 54 \times 86$ mm) were fitted with an air inlet and a water outlet. The air was pumped and sterilized through a 0.2 µm pore filter membrane, and water was supplied through a peristaltic pump at 2.4-6 l/h. The reason for this surprisingly high renewal rate was not given. Autoclaved leaves were inoculated with conidia and/or bacteria and submerged in the staining jar for 14 days. Five species of Ingoldian fungi and a *Fusarium* grew well and sporulated freely, the nutrients being provided exclusively by the leaves. Conidial counts were made directly on the leaves and not on the filtered outflowing water, where they would have been more accurate. This could have been due to conidial adherence to the walls of the staining jar. Sporulation rates through time were not provided.

B) Contact with nutrient solutions

B1. Unchanged media: these conditions do not seem to have been used, but such systems could be incorporated into experiments aimed at dilucidating the differences between the effects of nutrient supply and staling compound elimination.

B2. Renewed nutrient media

B2a. Periodic renewal of the nutrient media

Ciferri (1959) studied the fungal colonization of leaves by submerging them in an aerated mineral nutrient medium which was siphoned off and renewed three to five times a day. Although water in streams is known to carry nutrients, this is the first record we have of nutrient renewal in the laboratory culture, and apparently no new attempt was made until that of Suberkropp (1991), who designed «aeration chambers» for studying mycelial growth yields and decomposition and sporulation rates of Ingoldian fungi on leaf discs. Broad glass tubes covered by glass lids were provided at the bottom with an air inlet and a drainage tube. Incubations were at 15 °C in a dilute mineral salt solution (detailed above), which was drained every other day and replaced from the top. Air was pumped in at 80-100 mL/min. Conidia were produced for the entire length of the experiment (up to 29 days), presumably because of the periodic renewal of the medium. Any adherence of conidia to walls was apparently not noted. The technique requires that nutrient media have to be refilled in a sterile air-flow cabinet.

B2b. Continuous renewal of the media

B2b1. Perfusion in open air

We have recently attempted to quantify sporulation by means of an open air axenic perfusion technique, which eliminates the problem of conidial adherence. A carboy filled with a nutrient solution (0.01% malt extract in distilled water) was filted with a 1 mm diam. silicone tube leading to a hole in the lid of a culture flask containing a small amount of a non-volatile fixative (e.g. a concentrated toxic salt solution). A small hook was hung from the end of the silicone tube and one end of a 4×1 cm filter paper strip colonized with *Tricladium splendens* pierced through the hook. The nutrient solution dripped at *ca*. one drop/10s, which kept the colony permanently soaked. The fixed conidial suspension collected in the bottle was intended to be filtered for conidial counting. But most detached conidia unexpectedly remained clustered on the filter paper instead of falling with the drops (This effect may paralell water drops falling from trees, and this should now be checked). When the filter paper was submerged in a dish with water the conidia quickly dispersed in large numbers. Conidia were typical of the species and of a regular size, indicating adequate sporulation conditions. There had been apparently no adherence, either mutual or to the filter paper.

An automatic periodic flushing devise based on the principle of pipette cleaners and placed along the nutrient feeder tube could help remove the conidia intermittently and thus allow for quantitative harvesting. Alternatively devices based on removing the conidia by splashing off or by continuous vibration of the substratum

could also be attempted. If this were achieved, the use of inert materials such as glass fibre, instead of a degradable ones such as filter paper, should enable studies on the effect of nutrient choice and concentration in the liquid medium on relationships between mycelial growth and sporulation. The minimal rate of medium renewal needed for optimum sporulation could also be determined.

B2b2. Submersion

Marvanová (1977) successfully attempted to study a hyphomycetous parasite growing on conidia of *Anguillospora crassa* by placing these under a thin coverglass in a closed perfusion chamber through which a nutrient solution was passed. The chamber was not described but it seems to have been based on the same design as that used by Marvan *et al*. (1979) (cited by Marvanová & Bandoni 1987; see above).

Sterile systems are now being designed in our laboratory (fig. 1) with the aim of providing all those factors believed to induce or promote sporulation, either of mycelia on natural substrata or in pure culture (i.e.: constant forcible aeration, removal of metabolites, supply of dissolved nutrients, light and near UV irradiation) as well as a simple and efficient means of continuously harvesting conidia. One example of such a system is described here (Fig. 1):

The culture chamber (made to order by a commercial glass-blower) consists of a screw-capped test tube (100 × 15 mm) made of Pyrex glass, as it is transparent to near UV. Daylight fluorescent and lamps can then be installed parallel to the chamber. This is kept small to minimize the turnover time of the liquid medium, and it is narrow to reduce the recirculation of conidia in the medium.Two perforations are made in the wall immediately below the bakelite screw cap and glass tube inlets connected for the forced air and for the nutrient solution. The perforated base of the test tube (which operates in an inverted position) is connected to one end of a U-shaped outflow tube. The other end of this U-tube is pushed through a hole in the lid of a bottle containing a fixative (see above) where the conidial suspension will be collected. This end of the U-tube is lightly smeared on the inside with vaseline to avoid bubble bursting, which would project conidia against the sidewalls of the collecting bottle, where they would germinate and thus be lost for counting purposes. Air is forced into the culture chamber from an aquarium pump and sterilized by an on-line 0.2 (m filter membrane, or more simply by packing a 10 cm length of cottonwool into the silicone tubing. The filter membrane or cottonwool as well as the air pump are kept above the level of the culture chamber to avoid flooding in case the pump were to stop accidentally or during manipulation. As a further precaution, an aquarium-type one-way valve may also be fitted along the tube.

The nutrient solution (0.01% malt extract) can be forced into the culture chamber with a peristaltic pump, but to reduce, the cost gravity flow at the rate of 1 drop/10s from an inverted 5 l carboy with a constant head device is an alternative. The head device consists of a thin silicone tube running from a valve in the autoclavable screw-cap (Multifit Ltd.) of the carboy. The upper end of this outer tube is

packed with cottonwool to maintain sterility. Air bubbles will regularly enter the carboy as a vacuum is created in this as when the medium drips into the culture chamber.

The nutrient renewal rate is controlled by a clamp valve and also hydrostatically by raising or lowering the collection bottle on which the culture chamber rests. A glass rod on which the fungus will grow and sporulate is placed in the culture chamber. The carboy and culture chamber are connected and then autoclaved with the carboy in an upright position and its Multifit cap loosened to keep the medium from being sucked out when it is depressurized after autoclaving. After the nutrient medium has cooled overnight, the fungus is inoculated (in the laminar flow hood) onto the glass rod and then incubated in the culture chamber prior to submersion to allow for mycelial anchorage. The incubation time will depend on the growth rate of the species under study. Alternatively the rod may be colonized separately, for example in a Petri dish, prior to being introduced in the culture chamber.

When submerged in the aerated, continuously renewed medium in the culture chamber the mycelium will grow at rates dependent on those of air flow and nutrient concentration. (Rods of various lengths could also be used to study the effect of depth of mycelial submersion on sporulation). As soon as sporulation starts, the air bubbles will lift the conidia and carry them by surface tension into the collection bottle. For quantitative studies, aliquots of the conidial suspension may be periodically counted (e.g. by membrane filtration) and sporulation-time curves thus obtained. Total sporulation can also be correlated with the mycelial growth curve. If daughter colonies develop on the inside wall of the culture chamber, the difference in the dry weight of the chamber may be added to that of the rod.

Recent tests of this system with *Tricladium splendens* produced large numbers of conidia which were of normal and constant morphology. Sporulation proceeded until the carboy was emptied. Conidial harvesting seemed to be efficient at high aeration rates, but at lower ones some conidia were trapped on the inner sidewalls of the culture chamber.

Very high aeration rates have resulted in the production of a thick mycelial felt, which could eventually interfere with air, medium and conidial flow.

As happens with pellets formed in liquid culture, and assuming that sporulation only occurs on their surface, the ratio of mycelial surface (and thus of conidiophore numbers and conidial production) to mycelial biomass should drop with time; but that of quantity of sporulation to mycelial surface should be more constant. This last ratio is also being frequently used by various authors in the quantification of sporulation of Ingoldian fungi on leaf and agar discs in aeration systems.

Optimum nutrient concentrations and renewal and aeration rates have not yet been calculated but will probably vary with species. The system above should be adequate for application in physiological work as well as for studying field-colonized substrata. It should also be convenient for producing abundant sporulating material for microscopic observation in taxonomic work and for long-term cryo-preservation, as the surface of the mycelial felt may be easily sampled with sterile forceps.

B2b3. Passive diffusion

Dialysis bags have been used for some time in phyto- and bacterioplankton production studies in the field (e.g. Tóth 1980). Such non-degradable semipermanent membranes were thought to be probably efficient in enabling the diffusion in either direction of dissolved gases, ions and organic molecules assimilated or eliminated by fungi under submerged conditions. If this is true, and as the dialysis bag contents can be kept sterile, it should be possible to perform nutritional and sporulation tests with fungal cultures on inert or natural substrates in streams without interference from other organisms.

In a study on the effect of heavy metals on mycelial survival and decomposition rates of *Tricladium chaetocladium,* a preliminary test with dialysis bags was performed by M. Iles (1983, unpubl. data, available from E.D.) as an undergraduate project at the Univ. Exeter, UK. Colonized leaf discs were placed axenically in autoclaved 25 cm nylon dialysis tubes which were then heat-sealed at both ends and loosely hung (to provide some mixing in the medium through movement caused by stream turbulence) inside land drains. These were anchored lengthwise on the streambed. Treatments and controls were run in triplicate. The effect on sporulation rates was not the aim of the exercise, but after two weeks' submersion sporulation in the dialysis bags was profuse. Conidia were typical, of regular size and had not germinated, and no adherence was observed. The control in Iles' test consisted of colonized leaf discs placed in sterile stream water in submerged, screw-capped glass bottles.There was consequently some mixing, but no nutrient supply or elimination of gaseous or soluble metabolites. Other controls need to be established to further test the dyalysis tube technique. An obvious disadvantage of dialysis bags for experimental work is that periodic monitoring of sporulation would not be feasible without destructive sampling.

SUGGESTIONS FOR FUTURE RESEARCH

As evidenced from the little information which is now available on the various environmental conditions affecting sporulation in the Ingoldian fungus, especially with regard to water relations, a thorough study is required.

In addition, several possible areas for future research can be identified:

a) Laboratory studies, aimed at testing:

- — solid physical or chemical criteria for the distinction between aerial, interfacial and aquatic sporulation;
- — the use of aquatic techniques for spermatization of randomly paired cultures of fungi bearing synanamorphs, with the aim of establishing or confirming anamorph/teleomorph connections;
- — the effect of water activity of agar media and nutrient solutions on sporulation;

— optimization of nutrient renewal techniques for application in routine sporulation protocols;
— the sporulation dynamics of Ingoldian fungi as affected by interactions with other fungi, for example with regard to changes in temperature optima; interactions should also include those microorganisms believed to have a strong presence in aquatic ecosystems, i.e. lower fungi, possibly some *Mucoraceae,* algae, bacteria, actinomycetes, nematodes, rhizopods, etc;
— whether submerging cultures of aero-aquatic fungi yield further Ingoldian morphs (though there is probably a low chance for this, because the aero-aquatic type conidia would have already appeared on cultures isolated from aquatic conidia prior to their submersion);
— the extent and control of conidial adherence in harvesting techniques;
— the effect of light radiation (and especially near UV) on submerged or interface sporulation;
— the biological significance of aquatic production of anamorphs and synanamorphs (and possibly even teleomorphs) in extreme conditions such as the high acidity of some moorland waters;
— the effect of varying the levels of relative humidity on aerial sporulation in a range of Ingoldian fungi;
— the effect of nutrient renewal on sporulation in the case of restricted growth;
— open-air perfusion techniques *vs.* submerged ones;
— etc...

b) Field (combined with laboratory) studies aimed at testing, e.g.:

— the possible existence of a parallel group of «aquatic» or «amphibious» basidiomycetes, i.e. adapted to aquatic (asexual) sporulation and dispersal;
— the possible existence of a similar ecological group of coelomycetes;
— the *in situ* sporulation habits, if any, of soil and litter fungi in streams, (and whether they are active in decomposition);
— the response of conidial production to the presence of condensation water;
— whether there is a continuum in *in situ* sporulation requirements with regard to water relations of fungi collected along a transect from terrestrial to aquatic habitats, especially in dry climates;
— the extent to which Ingoldian fungi exploit the endophytic habitat in streams and its role in aquatic ecosystems;
— survival strategies (including sporulation habits) of Ingoldian fungi in temporary waters, especially in dry climates;
— whether the hyporrheos, sediment and riparian soils of streams and large rivers are colonized by the Ingoldian fungi and whether conidial production or dispersal occur there;
— the extent and duration of *in situ* conidial production, or its repression on submerged substrata in various seasons of the year;

— the spore composition of waters in spate in order to understand the response of both submerged and exposed mycelia of various groups of waterborne fungi to increased stream flow;
— the sporulation dynamics of Ingoldian and other aquatic fungi in water *vs.* different levels and chemical types of salinity;
— the effect of organic pollution on sporulation;
— the effect of hydrostatic pressure on sporulation;
— etc...

With regard to sporulation techniques, much of the information presented in this contribution has been gleaned from taxonomic and other publications where techniques have often been improvised or tested without proper controls. There is thus a need to follow this up with carefully designed experiments, after determining which environmental factors really play a significant role in submerged and interface sporulation.

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